

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau

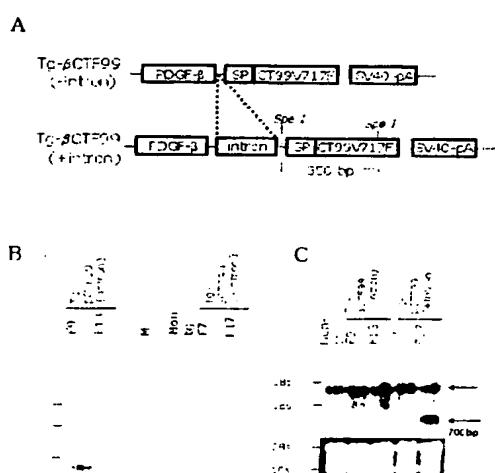


(43) International Publication Date  
12 January 2006 (12.01.2006)

PCT

(10) International Publication Number  
**WO 2006/004306 A1**

- (51) International Patent Classification<sup>7</sup>: C12N 15/63
- (21) International Application Number: PCT/KR2005/000969
- (22) International Filing Date: 1 April 2005 (01.04.2005)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
10-2004-0022562 1 April 2004 (01.04.2004) KR
- (71) Applicant (for all designated States except US): EWHA UNIVERSITY-INDUSTRY COLLABORATION FOUNDATION [KR/KR]; 11-1, Ewha University Daehyeon-dong Seodaemun-ku, Seoul 120-750 (KR).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): HAN, Pyung Lim [KR/KR]; #109-504 Hansin Chonggu Apt. Mok6-dong Yangcheon-ku, Seoul 158-759 (KR). LEE, Kang-Woo [KR/KR]; 822 Singil7-dong Yeongdeungpo-ku, Seoul 150-854 (KR). YANG, Sung-Don [KR/KR]; #107-304 DaelimDure Apt. Sinseong-dong Yusung-ku, Taejeon-si 305-720 (KR). SONG, Jin-Sook [KR/KR]; #115-1004 Hanbit Apt. Oun-dong Yusung-ku, Taejeon-si 305-755 (KR).
- (74) Agent: LEE, Won-Hee; 8th Fl. Sung-ji Heights II 642-16, Yoksam-dong Kangnam-ku, Seoul 135-080 (KR).
- (54) Title: TRANSGENIC MICE INDUCING ALZHEIMER'S DISEASE EXPRESSING MUTANT BETA CTFF99



**WO 2006/004306 A1**

(57) Abstract: The present invention is related to a transgenic animal inducing Alzheimer's disease. More particularly, the present invention is a vector for transformation of animal comprising a carboxyl-terminal fragments of mutant human beta amyloid protein which contains Indiana mutation (βCTF99) and a transgenic mouse inducing Alzheimer's disease prepared by microinjection of the same into a pronuclei of a fertilized oocyte. The transgenic mouse of the present invention exhibited clinical symptoms of Alzheimer's disease such as decreases of cognitive ability and memory, and increases of anxiety. Therefore, the transgenic mouse of the present invention will be a useful animal model for a research of Alzheimer's disease. Particularly, since the transgenic mouse of the present invention showed more remarkable decreases of cognitive ability than any other transgenic animal model for Alzheimer's disease known in the art, the transgenic mouse of the present invention can be used as an animal model for disease relating anxiety.

## [DESCRIPTION]

## [Invention Title]

**TRANSGENIC MICE INDUCING ALZHEIMER'S DISEASE  
EXPRESSING MUTANT BETACTF99**

5

## [Technical Field]

The present invention relates to a transgenic mouse with induced Alzheimer's disease pathology, more precisely, a transgenic mouse that shows Alzheimer's disease pathology induced by the insertion of a cDNA of a mutant human amyloid beta precursor protein into chromosomal DNA.

## [Background Art]

Increased production of  $\beta$ -amyloid peptide (referred 15 "A $\beta$ " hereinafter) has reported to be involved in the pathogenesis of Alzheimer's disease (AD). A $\beta$  can be produced by sequential action of  $\beta$ -secretase and  $\gamma$ -secretase inducing proteolytic cleavages of APP. Presenilin-1 (referred "PS1" hereinafter) may be a key 20 component of  $\gamma$ -secretase complex or regulate traffics of its' matrix (Esler WP and Wolfe MS, 2001, *Science*, 293:1449-1454). Thus, PS1 has been considered to be a therapeutic target for the treatment of AD and the delayed expression of AD symptoms (Esler WP and Wolfe MS, 2001, 25 *Science*, 293:1449-1454; Li YM et al., 2000, *Nature*,

405:689-694). However, it is still in doubt whether or not  $\gamma$ -secretase inhibitor activity is involved in the accumulation of  $\beta$ -CTF.

5       Recent studies with a conditional knockout strategy have circumvented the lethality of PS1 deficient mice and generated adult animals lacking PS1 specifically in the brain (Yu H et al., 2001, *Neuron*, 31:713-726; Dewachter I et al., 2002, *J. Neurosci*, 22:3445-3453). The study  
10 involving these double transgenic mice carrying both the PS1 conditional mutation and the APP<sub>v717i</sub> transgene revealed that the elimination of the  $\gamma$ -secretase activity provided by PS1 markedly reduced A $\beta$  production, plaque deposition and rescued impaired hippocampal LTP, but that  
15 it neither corrected the deficits in memory that APP<sub>v717i</sub> transgenic mice displayed nor stopped the progress of neurophysiological and pathogenic symptoms (Dewachter I et al., 2002, *J. Neurosci*, 22:3445-3453). Although the underlying mechanism has not yet been clearly elucidated,  
20 these studies show that the loss of  $\gamma$ -secretase activity in the brain leads to the severe accumulation of beta-CTF99, and raise the possibility that  $\beta$ CTF99 accumulation might cause cognitive deficits in the absence of plaque deposition in their double transgenic mice. It also  
25 raises the question as to whether or not other biochemical

impairments or behavioral alterations present in APP<sub>V717I</sub> transgenic mice can be reverted in their double transgenic mice. Regarding that PS1 has pleiotropic roles in brain cell functions including Notch (Naruse S et al., 1998, 5 *Neuron*, 21:1213-1221; Song W et al., 1999, *Proc. Natl. Acad. Sci. USA.*, 96:6959-6953) and N-cadherin processing (Marambaud P et al., 2003, *Cell*, 114:635-645), it needs to be answered whether or not the observed memory deficits of these double transgenic knockout mice were produced solely 10 by  $\beta$ CTF99.

More direct evidence for the *in vivo* role of  $\beta$ CTF99 is to be ascertained from studies with transgenic mice expressing  $\beta$ CTF99 in the brain. Eight research groups 15 have independently created transgenic mouse lines expressing various CTF forms of the human APP in the brain. Four of those lines showed either neuronal atrophy (Oster-Granite et al., 1996, *J. Neurosci.*, 16:6732-6741; Nalbantoglu J et al., 1997, *Science*, 387:500-505; Sato et 20 al., 1997, *Dement Geriatr Cogn Disord*, 8:296-307) or impaired learning (Nalbantoglu J et al., 1997, *Science*, 387:500-505; Berger-Sweeney J et al., 1999, *Brain Res Mol Brain Res*, 66:150-162; Laronde R et al., 2002, *Brain Res*, 956:36-44) at age 12 - 28 months, whereas the other four 25 lines did not display any obvious neuronal loss or

cognitive impairment (Sandhu *et al.*, 1991, *J Biol Chem.*, 266:21331-21334; Araki *et al.*, 1994, *Int. J. Exp. Clin. Invest.*, 2:100-106; Sberna *et al.*, 1998, *J. Neurochem.*, 71:723-731; Li *et al.*, 1999, *J. Neurochem.*, 72:2479-2487; 5 Rutten *et al.*, 2003, *Neurobiol Dis.*, 12: 110-120). Thus, the developed transgenic mice expressing CTFs showed conflicting results that ranged from no phenotype to AD-like pathogenesis. Accordingly, the *in vivo* role of  $\beta$ CTF99 remains elusive.

10

The present inventors have thus tried to establish an animal model for AD study, and finally prepared a transgenic mouse line bearing clinical symptoms and characteristics of AD. And further, the inventors have 15 completed the present invention by confirming that this newly created transgenic mouse clearly shows AD symptoms.

#### 【Disclosure】

#### 【Technical Problem】

20 The object of the present invention is to provide a transgenic vector that can be used to create transgenic mice showing AD pathology.

Another object of the present invention is to create a genetically stable transgenic mouse carrying the above 25 vector.

**【Technical Solution】**

To achieve the above objects, the present invention provides a transgenic vector that contains a gene coding a  
5 C-terminal fragment (CTF) of mutant human amyloid beta precursor protein (APP).

The present invention also provides a transgenic mouse that was produced by injection of the vector into the nucleus of a fertilized egg of mice, followed by  
10 transferring injected eggs into the oviduct of foster mothers to generate mice.

**【Advantageous Effects】**

The transgenic mouse of the present invention showed  
15 remarkable cognitive impairments compared to those of the wild type mouse both in the Morris water maze test and in passive avoidance test. In addition, the transgenic mouse showed highly increased anxiety compared to that of the wild type mouse in the elevated plus maze test, indicating  
20 that this AD animal presents AD symptoms more clearly than any other known AD animal models. Since the transgenic mouse of the present invention shows clear AD symptoms, this animal can be used as an animal model not only for studies of AD pathogenesis but also for studies on  
25 cognitive and anxiety impairments.

**【Description of Drawings】**

Fig. 1A is a schematic diagram showing the vectors 'PDGF- $\beta$  CTF99(V717F)-pA' and 'PDGF-intron- $\beta$  CTF99(V717F)-pA' for transformation constructed in the present invention.

Fig. 1B is a photograph of Southern blotting confirming the insertion of  $\beta$ CTF99(V717F) mutant gene in transgenic animals of the invented 'Tg- $\beta$ CTF99/B6(-intron)' and 'Tg- $\beta$ CTF99/B6(+intron)'. In the photograph, the arrow presents 350 bp  $\beta$ CTF99 fragment digested by SpeI.

Fig. 1C is a photograph of Northern blotting confirming the expression of  $\beta$ CTF99(V717F) mutant gene in transgenic mice of the invented 'Tg- $\beta$ CTF99/B6(-intron)' and 'Tg- $\beta$ CTF99/B6(+intron)'. In the photograph, the upper arrow presents internal  $\beta$ CTF99 transcript (3.5 kb) and the lower arrow presents mutant  $\beta$ CTF99 transcript (700 pb) of the present invention.

Fig. 2A is a set of a photograph of Western blotting confirming the production of  $\beta$ CTF99 protein in Tg- $\beta$ CTF99/B6 transgenic mice of the present invention (left

panel) and a graph showing the quantification of the production above (right panel). In the photograph of Western blotting, the upper and lower panels are prepared using  $\alpha$ CTF antibody and  $\beta$ CTF antibody, respectively. And in the graph, the data obtained from four different experimental groups are presented as the means  $\pm$  SEM.

Fig. 2B-C are photographs of immunohistological analysis investigating the expression of  $\beta$ CTF protein in cerebral cortex (CX) of Tg- $\beta$ CTF99/B6 transgenic mouse (C) of the present invention and their wild type mice(B).

Fig. 3A is a photograph of Western blotting measuring the expressions of p-JNK, p-c-Jun, JNK1, JNK2, JNK3, p-ERK, ERK, p-p38 and p38 $\alpha$  protein in the brain of Tg- $\beta$ CTF99/B6 transgenic mouse of the present invention.

Fig. 3B is a graph showing the expression levels of p-JNK (left panel) and p-c-Jun (right panel) in the brain of Tg- $\beta$ CTF99/B6 transgenic mice of the present invention. Data presented are the means  $\pm$  SEM of 7 and 4 independent experiments on 6 and 4 animals ( $n=4-6$ ) for p-JNK and p-c-Jun, respectively.

Fig. 4A is a set of a photograph (left panel) of

Western blotting measuring the expressions of Bcl-2, Bcl-x<sub>L</sub>, Bad and Bax proteins in the brain of Tg- $\beta$ CTF99/B6 transgenic mouse of the present invention at 14-15 months and a graph (right panel) is the results above presented 5 in relative expression levels. Each data obtained from 3 other experimental groups is presented as the means  $\pm$  SEM.

Fig. 4B is a photograph resulted from immunohistological analysis of the expressions of Bad and 10 Bax proteins in CA1, CA3 and DG regions of hippocampus (HP) in the brain of Tg- $\beta$ CTF99/B6 transgenic mouse at 14-15 months of the present invention, and a graph (right panel) is the results above presented in relative expression levels. In the photograph, the scale bar in the 15 upper panel represents 200  $\mu$ m and three scale bars in the lower panel represent 500  $\mu$ m each.

Fig. 5A is a photograph (left panel) of Western blotting measuring the expression of calbindin protein in 20 the brain of Tg- $\beta$ CTF99/B6 transgenic mouse at 15 months of the present invention and a graph (right panel) is the results above presented in relative expression levels. Each data cbtained from three independent experimental groups is presented as the means  $\pm$  SEM.

Fig. 5B is a photograph of immunohistological analysis measuring the expression of calbindin protein in CA1, CA3 and DG region of hippocampus (HP) in the brain of Tg- $\beta$ CTF99/B6 transgenic mouse at 15 months of the present invention, and a graph (right panel) is the results above presented in relative expression levels. The scale bar in the upper panel represents 200  $\mu\text{m}$  and the scale bars in the lower panel represent 500  $\mu\text{m}$  each.

Fig. 6A is a set of a photograph (left panel) of Western blotting measuring the expressions of CREB and phosphorylated-CREB proteins in the brain of Tg- $\beta$ CTF99/B6 transgenic mouse at 15 months of the present invention and a graph (right panel) is the results above presented in relative expression levels. In the graph, each data obtained from three independent experimental groups is presented as the means  $\pm$  SEM.

Fig. 6B-K are photographs of immunohistological analysis measuring the expressions of CREB and phosphorylated-CREB proteins in CA1 of hippocampus (HP), cerebral cortex (CX) and DG regions in the brain of Tg- $\beta$ CTF99/B6 transgenic mouse at 15 months of the present invention, and a graph (right panel) is the results above presented in relative expression levels. The scale bars

in panel C, E and K represent 50  $\mu\text{m}$  each.

Fig. 7A-H are photographs of immunohistological analysis measuring the expressions of Neu-N protein (A-D) 5 and MAP2 protein (E-H) in CA1 region of hippocampus (HP) and cerebral cortex (CX) of the brain of Tg- $\beta$ CTF99/B6 transgenic mouse of the present invention at 18 months and the wild type control mouse.

10 A: Prefrontal cortex of the wild type control mouse was stained with anti-Neu-N antibody,

B: Prefrontal cortex of Tg- $\beta$ CTF99/B6 transgenic mouse was stained with anti-Neu-N antibody,

C: Pyramidal cells of the wild type control mouse were stained with anti-Neu-N antibody,

15 D: Pyramidal cells of Tg- $\beta$ CTF99/B6 transgenic mouse were stained with anti-Neu-N antibody,

E: Prefrontal cortex of the wild type control mouse was stained with anti-MAP2 antibody,

20 F: Prefrontal cortex of Tg- $\beta$ CTF99/B6 transgenic mouse was stained with anti-MAP2 antibody,

G: CA1 of the wild type control mouse was stained with anti-MAP2 antibody,

H: CA1 of Tg- $\beta$ CTF99/B6 transgenic mouse was stained with anti-MAP2 antibody.

Fig. 7I is the result showing gradual neurodegeneration revealed by measuring the expression of Neu-N protein in the brains of Tg- $\beta$ CTF99/B6 transgenic mice at 12 and at 18 months of the present invention.

5

Fig. 8A is the result of the open field test showing the locomotor activities of Tg- $\beta$ CTF99/B6 transgenic mice at 7 and at 14 months of the present invention.

10

Fig. 8B is the result of the rota-rod test showing the locomotor activities of Tg- $\beta$ CTF99/B6 transgenic mice at 5.5 and at 11 months of the present invention. In the graph, the data obtained from 6-15 independent experimental groups are shown as the means  $\pm$  SEM.

15

Fig. 9A-B is the result of Morris water maze test. The latency to find a hidden platform was recorded to investigate cognitive impairments of Tg- $\beta$ CTF99/B6 transgenic mice at 7 months of age (A) and at 14 months of age (B) of the present invention. In the graph, \* indicates a difference at the  $p<0.05$  level in each group (Student's t-test). The data obtained from 6-8 independent experimental groups are presented as the means  $\pm$  SEM.

25

Fig. 9C shows the results of the Morris water maze

test showing swimming speed of animals to find a hidden platform, which was investigated to measure whether the transgenic animals of the present invention have any general motor function impairments. In the graph, \* 5 indicates a difference at the  $p<0.05$  level in each group (Student's t-test). The data obtained from 6-8 independent experimental groups are presented as the means  $\pm$  SEM.

Fig. 9D shows the results of the passive avoidance 10 test to investigate cognitive impairments of Tg- $\beta$  CTF99/B6 mice at 7 months and at 14 months of the present invention. In the graph, \* indicates a difference at the  $p<0.05$  level in each group (Student's t-test). The data obtained from 6-8 independent experimental groups are presented as the 15 means  $\pm$  SEM.

Fig. 10 shows the results of the elevated plus maze test to investigate anxiety state of Tg- $\beta$  CTF99/B6 mice at 13 months of age of the present invention. In the graph, \* 20 indicates a difference at the  $p<0.05$  level in each group (Student's t-test). The data obtained from 7-10 independent experimental groups are presented as the means  $\pm$  SEM.

25 【Best Mode】

Hereinafter, the present invention is described in detail.

The present invention provides a transgenic vector that contains a gene coding a C-terminal fragment of 5 mutant human amyloid beta precursor protein (APP), which can be used in the generation of AD mouse model.

The above C-terminal fragment of mutant human amyloid beta precursor protein (APP) includes the C-terminal fragment of APP bearing V717F mutation, which was 10 produced by the replacement of valine (V) with phenylalanine (F), which is represented by SEQ. ID. No 1. That is, the C-terminal fragment of APP bearing V717F mutation is preferred to have an amino acid sequence represented by SEQ. ID. No 3. In the preferred embodiment 15 of the present invention, the mutant  $\beta$ CTF99 represented by SEQ. ID. No 3, which was then named " $\beta$ CTF99(V717F)", was prepared by PCR using the second half of APP<sub>V717F</sub> cDNA represented by SEQ. ID. No 2 as a template.

It is also preferred for the transgenic vector of 20 the present invention, which includes PDGF- $\beta$  promoter, mutant  $\beta$ CTF99(V717F) encoding an amino acid sequence represented by SEQ. ID. No 3, and SV40 polyadenylation sequence. To increase translation efficiency, Kozac sequence was introduced in front of the above mutant 25  $\beta$ CTF99(V717F). The vector of the present invention was

designed to include PDGF- $\beta$  promoter, Kozac sequence, mutant  $\beta$ CTF99(V717F) represented by SEQ. ID. No 3 ( $\beta$ CTF99(V717F)) and SV40 polyadenylation sequence. The resulting vector was then named "PDGF- $\beta$ CTF99(V717F)-polyA" (see Fig. 1).

It is also preferred to construct the transgenic vector of the present invention, which has the intron B of the human  $\beta$ -globin gene inserted between PDGF- $\beta$  promoter and  $\beta$ CTF99(V717F). The introduction of the intron B gene of the human  $\beta$ -globin gene is to increase expression efficiency of the  $\beta$ CTF99(V717F) gene and transcription stability. So, in the present invention, the transgenic vector was constructed to include the PDGF- $\beta$  promoter, intron B of the human  $\beta$ -globin gene, Kozac sequence, mutant gene coding an amino acid sequence represented by SEQ. ID. No 3 ( $\beta$ CTF99(V717F)) and SV40 polyadenylation sequence. The resulting vector was named "PDGF-intron- $\beta$ CTF99(V717F)-polyA" (see Fig. 1A).

The present invention also provides a transgenic mouse with induced Alzheimer's disease prepared by inserting the vector of the invention into a mouse chromosome.

PDGF- $\beta$ CTF99(V717F)-polyA or PDGF-intron-  
25  $\beta$ CTF(V717F)-polyA transgenic vector is preferably

introduced into the pronucleus of mice to produce a transgenic mouse of the present invention, and PDGF-intron- $\beta$ CTF99(V717F)-polyA is more preferred. In the preferred embodiment of the present invention, PDGF-  
5  $\beta$ CTF99(V717F)-polyA or PDGF-intron- $\beta$ CTF99(V717F)-polyA transgenic vector was microinjected into the pronuclei of fertilized eggs prepared from inbred C75BL/6 mice, and the injected eggs were transplanted in surrogate mice. Comparison in expressions of  $\beta$ CTF99(V717F) mutant gene  
10 among the second generation produced from the surrogate mice and also offsprings produced by inbred was made. As a result, the expression of the mutant gene was much higher in transgenic mice transformed with PDGF-intron- $\beta$ CTF99(V717F)-polyA vector than in other transgenic mice  
15 transformed with the other vector. The result indicates that it is preferred to transform a mouse by the introduction of PDGF-intron- $\beta$ CTF99(V717F)-polyA vector to increase the insertion and expression efficiency of  $\beta$ CTF99(V717F) mutant gene of the present invention.

20 In the present invention, a transgenic mouse prepared by introducing PDGF-intron- $\beta$ CTF99(V717F)-polyA vector for transformation into nucleus of a fertilized egg was named "Tg- $\beta$ CTF/B6". After confirming that  $\beta$ CTF mutant gene was successfully inserted into a mouse and so  
25  $\beta$ CTF protein was expressed to the wanted level therein,

the present inventors deposited the transgenic mouse of the invention at Korean Collection for Type Cultures (KCTC) of Korea Research Institute of Bioscience and Biotechnology (KRIBB) on March 10, 2003 (Accession No: 5 KCTC 10609BP).

Gradual and age-dependent decrease of the expressions of calbindin and phosphorylated-CREB protein in hippocampus of transgenic mice with Alzheimer's disease induced in them(Tg- $\beta$ CTF/B6) were observed. In the meantime, neurodegeneration, motor coordination deficit, cognitive deficits and anxiety, which are characteristics shown in the brain of human AD patients, were increased.

In the preferred embodiment of the present invention, calbindin expression was significantly reduced in the hippocampus of the brain of Tg- $\beta$ CTF/B6 mice at 14-15 months of age (see Fig. 5). Calbindin is one of key components of calcium-binding proteins in the brain, along with parvalbumin and calretinin, which is presented as GABAergin and pyramidal neurons in various brain regions including frontal, temporal, entorhinal and hippocampus (Mikkonen et al., 1999, *Neuroscience*, 92:515-532). Calcium-binding proteins regulate intracellular calcium concentrations due to their calcium buffering capacity. 25 Altered intracellular calcium homeostasis may impair

normal cellular function and potentiate the cytotoxicity of neural cells (Berridge et al., 1998, *Neuron*, 21:13-26; Mattson, MP, 1998, *Trends Neurosci*, 21:53-57; Carafoli, E., 2002, *Proc. Natl. Acad. Sci USA*, 99:1115-1122). Mice lacking of calbindin showed impairments in spatial learning and LTP (Molinari S et al., 1996., *Proc. Natl. Acad. Sci USA*, 93:8028-8033). In aging or neurodegenerative brains, calbindin expression was reduced, leading to the pathologic changes (Iacopino A et al., 1990, *Proc. Natl. Acad. Sci USA*, 87:4078-4082; Leuba et al., 1998, *Exp Neurol.*, 152:278-291; Bu, J. et al., 2003, *Exp Neurol.*, 182:220-231). Although numbers of AD models have been developed as of today, no explanation has been given on the co-relation between the decrease of calbindin and the decrease of cognitive function except the recent report on transgenic mice expressing human APP mutation (Palop et al., 2003, *J. Neurosci.*, 100:9572-9577). And the present inventors have confirmed that the decrease of calcium-binding proteins is one reason for the cognitive deficits and other deficits that are characteristic features of AD patients.

The present inventors also observed the decrease of phosphorylated-CREB expression in hippocampus region of Tg- $\beta$ CTF/B6 mice (Fig. 6). Antisense oligodeoxynucleotide-mediated disruption of the CREB gene

in the hippocampus was found to impair long-term memory formation (Guzoski et al., 1997, *Proc. Natl. Acad. Sci USA*, 94:2693-2698), and a targeted mutation of the CREB $\beta$  isoform was associated with abnormal learning and memory 5 (Bourechuladze et al., 1994, *Cell*, 79:59-68; Blendy et al., 1996, *EMBO J.*, 15:1098-1106). On the other hand, increasing the level of CREB in the brain enhanced the formation of long-term memory (Josselyn et al., 2001, *J. Neurosci.*, 21:2404-2412). The decrease of phosphorylated-10 CREB expression in transgenic mice of the present invention resembles the result of investigation with AD patients. And, the reduced level of phosphorylated-CREB expression was confirmed to induce age-dependent cognitive impairment, neurodegeneration and the elevated anxiety. 15 The above results indicate that transgenic mice of the present invention can serve as a useful AD model.

Tg- $\beta$ CTF/B6 mice of the present invention showed similarities with the Tg2576+PS1P246L double transgenic mouse model (Savage et al., 2002, *J. Neurosci.*, 22:3376-20 3385), as both AD models showed increased JNK activation (see Fig. 3), a feature displayed by the human AD brain (Zhu et al., 2001, *J. Neurochem.*, 76:435-441; Savage et al., 2002, *J. Neurosci.*, 22:3376-3385). Single transgenic mouse Tg2576 or Tg-PS1P246L did not show any changes in 25 phosphorylated-JNK activation (Savage et al., 2002, *J.*

*Neurosci.*, 22:3376-3385), whereas transgenic mice of the present invention showed altered phosphorylated-JNK activation. In addition, transgenic mice of the present invention showed altered Bcl-2 family protein expressions 5 in the brain (see Fig. 4). The expressions of Bcl-2, Bad and Bax proteins were significantly increased, whereas Bcl-x<sub>L</sub> protein expression was reduced in transgenic mice of the present invention, indicating unbalanced Bcl-2 family protein expressions in the brain. The features of 10 Bcl-2 family protein expressions in transgenic mice of the present invention were similar to those in human AD patients (Nagy et al., 1997, *Neurobiol Aging*, 18:565-571; Kitamura et al., 1998, *Brain Res.*, 780:260-269), suggesting that the transgenic mice of the present 15 invention are very useful as an AD model.

The transgenic mice of the present invention (Tg- $\beta$  CTF/B6) showed motor coordination deficit, cognitive deficits and increased anxiety, which are characteristics shown in AD patients (see Fig. 8 ~ Fig. 10). In the 20 preferred embodiment of the present invention, in order to confirm whether or not Tg- $\beta$  CTF/B6 mice showed clinical symptoms of AD, open field test, rotar-rod test, Morris water maze test and passive avoidance test were performed to investigate cognitive capacity, and elevated plus maze 25 test was performed to investigate anxiety. As a result,

in open field test, there was no significant difference in locomotor activity between wild type and transgenic mice (see Fig. 8A). In the meantime, in rota rod test, locomotor activity of transgenic mice was a little reduced, 5 compared to that of the wild type mice, although the difference was not significant (see Fig. 8B). In Morris water maze test, transgenic mice showed cognitive deficits, resulting in impairment of memory (see Figs. 9A-C). In passive avoidance test, transgenic mice showed impairment 10 of memory retention, compared to that of the wild type mice (see Fig. 9D). Besides, in elevated plus maze test, notably increased anxiety was observed in transgenic mice, compared to that of the wild type mice (see Fig. 10). Thus, the transgenic mice of the present invention can be 15 effectively used as AD models because, as described above, they showed characteristic symptoms of AD such as memory deficits, cognitive deficits and increased anxiety.

#### 【Mode for Invention】

20 Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make

modifications and improvements within the spirit and scope of the present invention.

5           <Example 1> Preparation of human amyloid beta precursor protein cDNA and βCTF99 mutant gene

<-1> Preparation of human amyloid beta precursor protein cDNA

The cDNA coding human amyloid beta precursor protein (referred 'APP' hereinafter) was prepared by PCR using 10 Marathon-Ready cDNA library (Clontech, Palo Alto, CA, USA) constructed from human brain. The cDNA could not be amplified at once because the size of its open reading frame (ORF) was about 2.3 kb, taking APP770 as standard. Thus, the first half of the cDNA was amplified by using 15 primer sets of app-1f primer represented by SEQ. ID. No 6 (5'-gcaagggtcgcqatgctgccggttg-3', the underlined part presented Nru I restriction enzyme recognition site) and app-2r primer represented by SEQ. ID. No 9 (5'-gacattctctcggtgcttggcc-3'), and the resulting product 20 was digested with Nru I and Xho I. The product was inserted into Sma I and Xho I restriction enzyme recognition sites of pBluescript II KS vector (Stratagene, USA). The second half of the cDNA was amplified by using primer sets of app-2f primer represented by SEQ. ID. No 8 (5'-cctacaacagcagccagtacccctc-3') and app-1r primer 25

represented by SEQ. ID. No 7 (5'-gggggactagttctgcacatcgctc-3', the underlined part presented *Spe* I restriction enzyme recognition site), followed by digesting with *Spe* I and *Xho* I. Then, the resulting products were inserted into 5 *Spe* I and *Xho* I restriction enzyme recognition sites of pBluescript II KS vector. The DNA fragments produced by digesting pBluescript II KS vector bearing the first half of the cDNA with *BamH* I and *Xho* I and the other DNA fragments produced by digesting pBluescript II KS vector 10 bearing the second half of the cDNA with *Xba* I and *Xho* I were fused together with pBluescript II KS vector predigested with *Xba* I and *BamH* I, leading to the preparation of a vector construct carrying the full length 15 of APP cDNA. In the meantime, three different isoforms were produced, according to the numbers of amino acid residues of coded protein, from the same gene of human beta amyloid by selective splicing. So, according to the numbers of amino acid residues, APP cDNA carries three different isoforms, that is, APP770, APP751 and APP695. 20 The DNA sequences of the cloned cDNA were analyzed. As a result, among those three isoforms, the cloned cDNA was confirmed to be APP751 cDNA represented by SEQ. ID. No 1 coding APP751 (represented by SEQ. ID. No 2).

25 <1-2> Preparation of APP751 mutant gene

"V717F mutation (mutation in APP770 isoform induced by the replacement of valine, the 717<sup>th</sup> amino acid, with phenylalanine)" was introduced into APP751 cDNA by PCR. Particularly, PCR was performed by using pBluescript II KS vector carrying the second half of the APP751 cDNA produced in the above <Example 1-1> as a template with primer sets of app-2f primer represented by SEQ. ID. No 8 and app-717-r primer represented by SEQ. ID. No 11 (5'-caaggtgatgaagatcactgtcgc-3') with the 32 cycles of denaturation at 95°C for 1 minute, primer annealing at 57°C for 40 seconds and extension at 72°C for 1 minute. And the resulting product was used for another PCR under the same conditions as described above by using primer sets of app717-f primer represented by SEQ. ID. No 12 (5'-gcgacagtgatcttcatcaccttg-3') and app-1r primer represented by SEQ. ID. No 7 this time. The PCR products from the two PCR above had "V171F mutation". So, the two PCR products were separated, slowly cooled down, extended with Klenow enzyme and then fused into one fragment. The fragment was digested with *Xho* I and *Spe* I by taking advantage of *Xho* I restriction enzyme recognition site of app-2f primer and *Spe* I restriction enzyme recognition site of app-1r primer, which were inserted into pBluescript II KS vector which was also digested with *Xho* I and *Spe* I ahead of time and the second half of APP cDNA was inserted in, leading to

the preparation of the mutant second half APP751 cDNA. In the meantime, the mutated DNA fragment prepared above was used to replace the corresponding region of pBluescript II KS vector where the full length of APP751 cDNA was inserted, resulting in APP751 mutant cDNA represented by SEQ. ID. No 3, which encodes a protein represented by SEQ. ID. No 4, and then named "hAPP(V717F)". The nucleotide sequence of the hAPP(V717F) mutant gene was confirmed by DNA sequencing.

10

<1-3> Preparation of  $\beta$ CTF99 mutant gene

The present inventors tried to prepare a protein, which includes V717F mutation in the 717<sup>th</sup> amino acid region of the human amyloid beta precursor protein represented by SEQ. ID. No 1 and contains the C-terminal amino acid sequence. Particularly, the C-terminal fragment (672<sup>nd</sup> - 751<sup>st</sup>) was amplified by PCR using cDNA (SEQ. ID. No 3) coding APP751<sub>V717F</sub> protein (SEQ. ID. No 4), which induces Indiana mutation in APP protein represented by SEQ. ID. No 2, as a template, resulting in a mutant gene. PCR was performed by using the vector containing hAPP(V717F) cDNA prepared in the above <Example 1-2> as a template with primer sets of app99f primer represented by SEQ. ID. No 24 (5'-cgaattcgatgcagaattcc-3') and appr-1r primer represented by SEQ. ID. No 7 with 32 cycles of

denaturation at 95°C for 1 minute, primer annealing at 57°C for 40 seconds and extension at 72°C for 1 minute. The PCR product was digested with *EcoR* I and *Spe* I, which was linked to pBluscriptII KS vector (Stratagene) pre-digested  
5 with *EcoR* I and *Spe* I. Thus, the C-terminal of APP began to carry mutation, and the mutant gene was represented by SEQ. ID. No 5 and coded a protein represented by SEQ. ID. No 10. The resulting mutant gene was then named " $\beta$ CTF99".  
10 The nucleotide sequence of the mutant gene was confirmed by DNA sequencing.

In order to insert signal peptide in the above mutant gene, signal peptide region was amplified by PCR using pKS-aap696-1/2 vector bearing signal peptide as a template. The PCR was performed by using primer sets of  
15 app-sig-1f primer, represented by SEQ. ID. No 22, having *Bgl* II recognition site and app-sig-1r primer, represented by SEQ. ID. No 23, having *EcoR* I recognition site, with 32 cycles of denaturation at 95°C for 1 minute, primer annealing at 55°C for 1 minute and extension at 72°C for 1  
20 minute. The PCR product was digested with *Bgl* II, linearized by Klenow enzyme, digested with *EcoR* I, and then subcloned into *EcoR* I digesting region of pBluescript II KS vector (Stratagene) digested with *BamH* I, linearized by Klenow enzyme and digested with *EcoR* I.

25 In order to enhance translation efficiency of the

signal peptide, PCR was performed by using pBluescript II KS vector harboring the signal peptide as a template with primer sets of app-koz-f primer represented by SEQ. ID. No 15 having *Xba* I recognition site and Kozac sequence (GACC) 5 and app-koz-r primer represented by SEQ. ID. No 21 having *Not* I recognition site, followed by insertion of Kozac sequence (GACC) in front of starting codon (ATG) of the signal peptide. The PCR product was digested with *Xba* I and *Not* I, which was fused into pBluescript II KS digested 10 with *Xba* I and *Not* I, resulting in the construction of pKS-kozappsig vector. The vector was digested with *EcoRI* and the constructed  $\beta$ CTF99 vector was digested with *EcoRI*. The both digested products were fused, leading to the preparation of a vector producing a fusion protein where 15 signal peptide and  $\beta$ CTF99 were fused. So, the recombinant protein containing Kozac sequence, signal peptide and a gene coding  $\beta$ CTF99 protein in which Indiana mutation was induced was prepared and named " $\beta$ CTF99(V717F)".

20 <Example 2> Construction of an expression cassette containing  $\beta$ CTF99(V717F) mutant gene for transgenic animal

In order to prepare an AD animal model, an expression cassette for transformation containing 25  $\beta$ CTF99(V717F) mutant gene was constructed. Particularly,

pGK-neo-PA vector (Lee et al., *J. Neurosci.*, 2002, 15:7931-7940) was amplified using primer sets of SV40pA-f primer represented by SEQ. ID. No 13 (5'-tccccgcggtccagacatgataagatacattga-3', the underlined part presented *Sac* II restriction enzyme recognition site) and SV40pA-r primer represented by SEQ. ID. No 14 (5'-gttccgagctcataatcagccataaccacatttg-3', the underlined part presented *Sac* I restriction enzyme recognition site), resulting in 247 bp sized SV40-pA fragment for polyadenylation signal of the mutant gene. Then, the fragment was digested with *Sac* II and *Sac* I, which was inserted into pBluescript II KS vector. PsisCAT6a vector (Sasahara, M. et al., *Cell*, 1991, 64(1):217-27) was digested with *Xba* I, linearized with Klenow enzyme, and digested with *Hind* III, resulting in human platelet-derived growth factor-beta (PDGF-beta) promoter fragment. The obtained PDGF-beta promoter fragment was inserted into pBluescript II KS vector that was digested with *Sal* I, linearized with Klenow enzyme and then digested again with *Hind* III. The pBluescript II KS vector bearing the above PDGF-beta promoter fragment was digested with *Kpn* I and *Hind* III, resulting in 1.5 kb sized PDGF-beta promoter fragment. And the fragment was inserted into *Kpn* I and *Hind* III recognition sites of pBlescript II KS vector containing SV40 pA region. The resulting vector, thus,

has a structure that has PDGF-beta promoter and SV40-pA region respectively at each side of multicloning site of pBluescript II KS vector. In the vector,  $\beta$ CTF99(V717F) mutant gene containing Kozac sequence (GACC) in front of starting codon, prepared in the above <Example 1>, was inserted, resulting in an expression cassette for transformation. Finally, the expression cassette was constructed to possess PDGF- $\beta$  promoter- $\beta$ CTF99(V717F)-pA in that order, and named "PDGF- $\beta$ CTF99(V717F)-pA" (Fig. 1A).

<Example 3> Construction of an expression cassette containing intron and  $\beta$ CTF99(V717F) mutant gene for transgenic animal

The intron elevates expression efficiency of a mutant gene and increases transcription stability. Thus, in order to introduce a mutant gene into an animal model effectively, the present inventors introduced the intron B gene (918 bp) (Choi et al., *Molecular and cellular biology*, June 1991, p.3070-3074; Palmiter et al., *PNAS*, 1991, 88:478-482) of human  $\beta$ -globin gene into the expression cassette prepared in the above <Example 2>. Precisely, the intron B of human  $\beta$  globin gene was amplified by PCR using the primers of hglob-f represented by SEQ. ID. No 16 and hglob-r represented by SEQ. ID. No 17 and genomic DNA,

which was obtained from the human neuroblastoma cell line SH-SY5Y, as a template. The amplified intron B gene product derived from human  $\beta$ -globin gene was sub-cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), which 5 was inserted between PDGF- $\beta$  promoter gene of PDGF- $\beta$ CTF99(V717F)-pA expression cassette constructed in the above <Example 2> and  $\beta$ CTF99(V717F) mutant gene. The resulting expression vector for transformation was named "PDGF- $\beta$ CTF99(V717F)-pA" (Fig. 1A).

10

<Example 4> Generation of transgenic animals

PDGF- $\beta$ CTF99(V717F)-pA expression cassette constructed in the above <Example 2> and PDGF-intron- $\beta$ CTF99(V717F)-pA expression cassette constructed in the 15 above <Example 3> were digested with a restriction enzyme (*BssHII*), resulting in 3.1 kb sized linearized fragment. The product was microinjected into the pronuclei of fertilized eggs prepared from inbred C57BL/6 mice. After the microinjection, the fertilized eggs were transferred 20 to the oviduct of pseudopregnant female (ICR) mice. The methods for transformation of animals used in the present invention including microinjection were in accordance with the conventional methods (Games et al., *Nature*, 1995; Hisao et al., *Science*, 1996).

25

<Example 5> Confirmation of the insertion of a mutant gene into chromosomal DNA

Genomic DNA was extracted from the tails of F1 mice generated from the animal transformation procedure 5 performed in the above <Example 4>, and PCR was performed to confirm whether or not a mutant gene was rightly inserted into nuclei of fertilized eggs. Particularly, PCR was performed with primer sets of trapp-fs primer represented by SEQ. ID. No 18 and trapp-r1 primer 10 represented by SEQ. ID. No 19, in order to investigate the insertion of a mutant gene into the F1 mice generated by using PDGF- $\beta$ CTF99(V717F)-pA expression cassette excluding intron. In the meantime, another PCR was performed with primer sets of trint-f1 primer represented by SEQ. ID. No 20 and sv40pA-r primer represented by SEQ. ID. No 14, in 15 order to investigate the insertion of the mutant gene in the F1 mice generated by using an expression cassette including intron (PDGF-intron- $\beta$ CTF99(V717F)-pA).

20 As a result, among the F1 mice generated by the introduction of an expression cassette excluding intron (PDGF- $\beta$ CTF99(V717F)-pA), 16 mice were confirmed to bear the expression cassette. In the case of F1 mice generated by the introduction of an expression cassette including 25 intron (PDGF-intron- $\beta$ CTF99(V717F)-pA), only 2 mice were

confirmed to bear the expression cassette.

Southern blot analysis was also performed to confirm the introduction of the expression cassette of the present  
5 invention. Precisely, genomic DNA was extracted from the tails of F1 mice generated from the animal transformation procedure taken in the above <Example 4>, and then 15 µg of the genomic DNA was digested with restriction enzyme Spe I. The resulting products were electrophoresed on  
10 agarose gel, and then transferred onto nitrocellulose membrane. Hybridization was performed using a <sup>32</sup>P-labeled probe prepared from the 350 bp SpeI fragment at the C-terminus of APP cDNA, and the results were developed on X-ray film.

15

The results resembled those of the above PCR with the genomic DNA, that is, among the F1 mice generated by the introduction of an expression cassette excluding intron (PDGF- $\beta$ CTF99(V717F)-pA), 16 mice were confirmed to  
20 bear the expression cassette. In the case of those F1 mice generated by the introduction of an expression cassette including intron (PDGF-intron- $\beta$ CTF99(V717F)-pA), only 2 mice were confirmed to bear the expression cassette (Fig. 1B).

25

The transgenic mice which were confirmed by genomic

PCR and Southern blotting, to bear the  $\beta$ CTF(V717F) mutant gene of the present invention were inbred with C57BL/6 mice.

5       <Example 6> Investigation of the transgene expression

In order to confirm whether or not  $\beta$ CTF99(V717F) mutant gene was successfully introduced and expressed in the transgenic mice of the present invention, total RNA was prepared from the brains of transgenic mice, followed  
10 by Northern blotting. Northern blot analysis was performed according to the method of Lee, et al. (Lee et al., J Neurosci, 2002, 15:7931-7940). Precisely, total RNA was prepared from the brains of wild type and transgenic mice at 2 months, which were confirmed to have  
15  $\beta$ CTF99(V717F) mutant gene transduced in the above <Example 4> and <Example 5>. Trizol reagent (Sigma, St. Louis, MO, USA) was used for the extraction of the total RNA. A membrane blot carrying 30  $\mu$ g of total RNA was prepared after separating on denaturing agarose gel (1% agarose, 6.2% formaldehyde in 1 $\times$  MOPS), and hybridized  
20 with a  $^{32}$ P-labeled probe prepared from the *SpeI*-digested fragment (350 bp) of CTF99, which was also used in the above <Example 5> for Southern blot analysis, and then the results were developed on X-ray film. The probe was able  
25 to recognize both internal APP transcript and

$\beta$  CTF99(V717F) mutant transcript.

As a result, the expression of APP mutant transcript was much higher as a whole than that of the endogenous APP transcript in transgenic mice, regardless of that  $\beta$  CTF99(V717F) mutant gene transforming a mouse included intron or not. In particular, the expression of  $\beta$  CTF99(V717F) was especially higher in transgenic mice (F17 mice) bearing  $\beta$  CTF99(V717F) mutant gene harboring 10 intron (Fig. 1C). And those transgenic mice showing the high expression of  $\beta$  CTF99(V717F) mutant gene including intron were named "Tg- $\beta$  CTF99/B6", which were, from then on, used for further experiments of the present invention.

The transgenic mouse Tg- $\beta$  CTF99/B6 was deposited at 15 Korean Collection for Type Cultures (KCTC) of Korea Research Institute of Bioscience and Biotechnology (KRIBB) on March 10, 2003 (Accession No: KCTC 10609BP).

<Example 7> Protein production by the transgene

It was confirmed in the above <Example 6> that Tg- $\beta$  CTF99/B6 mice of the present invention expressed  $\beta$  CTF99(V717F) mutant gene successfully. In order to 20 investigate the possibility of protein production by the expressed gene, total protein was extracted from the brains of wild type controls and Tg- $\beta$  CTF99/B6 mice at 4 - 25

5 months, followed by Western blotting. The Western blot analysis was performed according to the method of Lee, et al. (Lee et al., *Brain Res Mol Brain Res*, 1999, 70:116-124). Particularly, mouse brain tissue was homogenized in 5 4°C lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) containing 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor (Complete<sup>TM</sup>; Roche, Mannheim, Germany). Centrifugation was performed with the homogenized brain tissue samples at 10 13,000 rpm, for 20 minutes at 4°C to obtain supernatant. The protein in the supernatant was quantified by BCA quantification kit (Sigma, St. Louis, MO, USA). Each lane was loaded with 30 µg of the protein, and acrylamide gel electrophoresis was carried out. The separated proteins 15 were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) and the membranes were blocked with 5% non-fat dry milk, 2% BSA, 4% FBS, 4% horse serum, 4% goat serum in Tris-buffered saline and 0.1% Tween 20. Two βCTF-specific polyclonal antibodies commonly detected the endogenous ~12 20 kD βCTF99 (A8717; Sigma, St. Louis MO, USA) and ~10 kD α CTF83(p3) (51-2700; Zymed, San Francisco, CA, USA) fragments were used for the Western blot analysis to confirm the production of the βCTF99(V717F) mutant protein. The βCTF99 and αCTF83 are proteins generated by 25 β-secretase and α-secretase, respectively, in the brains

of non-transgenic controls. Immunoblots were detected using ECL detecting reagents (Santa Cruz, CA, USA).

For the detection of  $\beta$ CTF99, the brain tissues were  
5 homogenized in 1:10 (g/vol) Tris-buffered saline (TBS)  
containing 50 mM Tris-HCl (pH 8.0), 175 mM NaCl, 5 mM EDTA,  
2 mM phenylmethylsulfonyl fluoride, and a protease  
inhibitor cocktail (Complete<sup>TM</sup>; Roche, Mannheim, Germany).  
Fifty  $\mu$ g of the protein sample were mixed with an equal  
10 volume of 2 $\times$  Laemmli sample buffer containing 10%  $\beta$ -  
mercaptoethanol, boiled for 10 min, and then  
electrophoresed on 16.5% Tris/tricine agarose gel as  
described (Li et al., 1999). After being transferred onto  
PVDF membranes, the resolved proteins were probed with  
15 polyclonal anti-CTF. Immunoblots were detected using ECL  
detection reagents.

As a result, in the Tg- $\beta$ CTF99/B6 brain at 4-5 months,  
the amounts of  $\beta$ CTF99 and  $\alpha$ CTF83 were notably up-  
20 regulated. Densitometric measurements of the CTFs using  
computer-assisted imaging software indicated that the  
expression levels of  $\beta$ CTF99 and  $\alpha$ CTF83 were  $2.63 \pm 0.37$   
and  $2.61 \pm 0.2$  fold that of endogenous  $\beta$ CTF99 and  $\alpha$ CTF83  
(Fig. 2A).

<Example 8> Immunohistochemical analysis of the brains of transgenic mice

For immunohistochemical experiments, the mice were perfused with 0.9% saline through ascending aorta, and 5 then perfused again with 4% paraformaldehyde in 0.1 M phosphate buffer (referred "PB" hereinafter, pH 7.4). The brain was removed and fixed in the fixative at 4°C. The fixed brain was coronally cut into 40  $\mu\text{m}$ -thick sections with a vibratome. The sections were reacted in 3% 10 hydrogen peroxide solution dissolved in 0.1 M PB (pH 7.4) for 30 minutes and washed with PB. The sections were blocked by 5% normal goat serum, 2% BSA and 2% FBS for 2 hours at room temperature. The primary antibody was added to the blocking buffer, which was left at 4°C for 15 overnight for reaction. After washing with PB solution, the secondary antibody, which was biotinylated by being diluted 1:200 fold, was added. Then, 1:100 fold diluted avidin and biotinylated HRP complex (Vector Laboratories, Burlingame, CA) were also added for one more hour reaction. 20 0.05% 3,3'-diaminobenzidine and 0.001% hydrogen peroxide in 0.1 M Tris (pH 7.4) were used for the color development. Cerebral cortex (referred "CX" hereinafter), pyramidal cells of CA1-CA3 regions (referred "CA1"- "CA3" hereinafter), hippocampus (referred "HP" hereinafter) and 25 dentate gyrus (referred "DG" hereinafter) were used for

the analysis.

As a result, increased expression of  $\beta$ CTF99 protein was observed in neuronal cells of broad brain regions  
5 including cerebral cortex in Tg- $\beta$ CTF/B6 mice of the present invention (Fig. 2B-C). However, plaque like-A $\beta$  deposition was not found in the brains of Tg- $\beta$ CTF/B6 mice at upto 18 months. And, approximately 92% of the Tg- $\beta$ CTF/B6 mice survived until at least 480 days, suggesting  
10 that those transgenic mice can survive longer than the conventional transgenic mice, eventhough the lethality is elevated compared to that of the wild type control mice. Thus, the transgenic mice of the present invention are much effective as animal models.

15

<Example 9> Expressions of other proteins in the brains of transgenic mice

Expressions of other proteins that might be affected by  $\beta$ CTF99(V717F) mutant gene introduced into Tg- $\beta$ CTF99/B6  
20 mice of the present invention were investigated. Particularly, Western blot analysis was performed with brain tissues by the same way as described earlier in the  
<Example 7>. The antibodies used for the analysis were anti-phospho-JNK antibody (9251S; Cell Signaling, Beverly,  
25 MA, USA), anti-phospho-c-Jun antibody (9261S; Cell

Signaling), anti-phosphc-p38 antibody (9211S; Cell Signaling), anti-JNK3 antibody (06-749; Upstate Biotechnology, Lake placid, NY, USA), anti-CREB antibody (Upstate Biotechnology), anti-phospho-CREB antibody (Upstate Biotechnology), anti-MAP2 antibody (Upstate Biotechnology), anti-calbindin antibody (C9848; Sigma, St. Louis, MO, USA), anti-parvalbumin (P3088; Sigma), anti-calretinin antibody (AB5054; Chemi-Con, Temecula, CA, USA), anti-JNK1 antibody (15701A; Pharmingen, San Diego, CA, USA), anti-JNK2 antibody (sc-572; Santa Cruz Bio-Technology, Santa Cruz, CA, USA), anti-phospho-ERK antibody (sc-7383; Santa Cruz Bio-Technology), anti-ERK antibody (sc-154; Santa Cruz Bio-Technology), anti-Bcl-2 antibody (sc-783; Santa Cruz Bio-Technology), anti-Bad antibody (sc-942-G), anti-Bax antibody (sc-6236; Santa Cruz Bio-Technology), and anti-Bcl-xL (sc-7195; Santa Cruz Bio-Technology).

Recent reports indicate that the human AD brain shows phospho-JNK up-regulation (Zhu et al., 2001, *J Neurochem.*, 76:435-441; Savage et al., 2002, *J Neurosci.*, 22: 3376-3385). Thus, the present inventors performed Western blot analysis to examine the expression of phospho-JNK protein in the brain of the transgenic mouse of the present invention. As a result, the expressions of

phospho-JNK protein and phospho-c-Jun protein in the brain of Tg- $\beta$ CTF99/B6 at 15 months were higher than those of age-matched wild type controls, whereas the expressions of JNK1, JNK2, JNK3, phospho-ERK and phospho-p38 were not 5 significantly changed (Fig. 3).

Bcl-2 and Bcl-xL proteins are anti-apoptotic whereas Bax and Bad proteins are pro-apoptotic. And these are B-cell leukemia-2 (Bcl-2) family proteins (Davies et al., 1995, *Trend Neurosci.*, 18:355-358). The present inventors 10 also performed Western blot analysis to detect the change of expression level of Bcl-2 family protein in the brain of the transgenic mice. As a result, the expressions of Bcl-2, Bad and Bax were significantly elevated, whereas Bcl-2-xL expression was attenuated in Tg- $\beta$ CTF99/B6 brain 15 at 14-16 months (Fig. 4A). The result indicates that the expression of Bcl protein is affected by the insertion of  $\beta$ CTF99(Ld) mutant gene of the present invention. Based on the result, immunohistochemical analysis was performed to examine the expressions of Bad and Bax in CA1 region, 20 the pyramidal cell layer of the hippocampus, in analogy to the procedure as described in the <Example 8>. Consistent with the results of Western blot analysis above, the expressions of Bad and Bax proteins in CA1 region were increased (Fig. 4B).

25 It has been known that AD brain shows the increased

expression of calcium-binding proteins (Anthony et al., 1990, Proc. Natl. Acad. Sci USA., 87:4078-4082; Mikkonen et al., 1999, *Neuroscience*, 92:515-532; Bu et al., 2003, *Exp Neurol.*, 182:220-231). Thus, the expressions of 5 calcium-binding proteins such as calbindin, parvalbumin and calretinin were investigated by Western blot and immunohistochemical assay. As a result, calbindin expression was reduced in hippocampus, CA1, CA3 and DG regions of Tg- $\beta$ CTF99/B6 at 14-16 months, compared to that 10 of the wild type controls (Fig. 5A-Western blot, Fig. 5B-Immunohistochemical assay). Calbindin expression was not detected in the brains of Tg- $\beta$ CTF99/B6 at 4-5 months. In the meantime, parvalbumin and calretinin expressions were not much different from those of wild type controls.

15       Recent reports indicate that the phospho-CREB level is reduced in the AD brain, which is nothing to do with the variations of total CREB protein level, though (Yamamoto-Sasaki et al., 1999, *J Neurosci.*, 22:1858-1867). Especially, the increased expression of CREB protein 20 during neuronal activity induces synaptic plasticity, in particular hippocampus-based memory retention (Mayford et al., 1999, *Trends Genet.*, 15:463-470; Colombo et al., 2003, *J Neurosci.*, 23:3547-3554; Viola et al., 2000, *J Neurosci.*, 20: RC112 (1-5)). Accordingly, the present inventors 25 performed Western blot and immunohistochemical analysis to

examine phospho-CREB protein expression at transgenic mice of the present invention. As a result, total CREB protein expression was not changed in the brain of Tg- $\beta$ CTF99/B6 at 14-16 months, whereas phospho-CREB protein expression 5 was reduced in hippocampus, CA1 and CX regions of the transgenic mice, compared to that of the wild type controls (Fig. 6A-Western blot, Fig. 6B-K-Immunohistochemical analysis). However, phospho-CREB protein expression in the brain of Tg- $\beta$ CTF99/B6 at 5-7 10 months was similar to that of wild type controls.

In order to examine the possibility of neuronal loss by  $\beta$ CTF99(V717F) mutant gene of the present invention, the expression of neuron-specific marker MAP-2 protein was measured. As a result, the expression of MAP-2 protein 15 was reduced in CX and hippocampus CA1 region of the brain of Tg- $\beta$ CTF99/B6 at 15-18 months, indicating that the mutant gene had influence on neuronal formation (Fig. 7A-H). However, the level of the protein in the brain of the transgenic mouse at 7 months was not much different from 20 that of a wild type control.

In order to examine the possibility of neuronal degeneration by  $\beta$ CTF99(Ld) mutant gene of the present invention, the expression of Neu protein was investigated. As a result, neuronal cell density was approximately 5-10% 25 reduced at the transgenic mice at 11-12 months, which went

further to 25% reduction at 18 months. The result indicates that  $\beta$ CTF99(Ld) mutant gene of the present invention induces gradual neuronal degeneration(Fig. 7I).

5       <Example 10> Cognitive function of the transgenic mice

Histopathological characteristics of AD brain are (1) the deposition of extracellular senile plaques, (2) the formation of intracellular neurofibrillary tangle, (3) the degeneration of axons and synapses, and neuronal loss, 10 and (4) malfunction of the brain by neuronal loss, which are all detectable by histological test. In particular, cognitive deficits are the most characteristic and important morphological and clinical symptom. Thus, it is important for an AD animal model to show not only 15 histological characteristics including senile plaques deposition, but also, in fact more importantly, cognitive deficits. The present inventors performed Morris water maze test, passive avoidance test, and open field test to judge the cognitive deficits in candidates for AD models.

20       Mice were housed in cages in a temperature- and humidity-controlled environment with a 12 hour-light/dark cycle (light switched on at 7 a.m.). All animals were handled in accordance with the animal care guideline of Ewha Womans University School of Medicine. To track the 25 animals' behavior, a computerized video-tracking system

(SMART; Panlab S. I., Barcelona, Spain) was used.

Two-sample comparisons were carried out using the Student t-test, while multiple comparisons were made using one-way ANOVA followed by the Newman-Keuls multiple range test. All data were presented as the means $\pm$ S.E.M. The statistical differences were accepted at the 5% level unless otherwise indicated.

<10-1> Open field test

10 Locomotor activity was measured in the open field of a white Plexiglas chamber (45×45×45 cm). Illumination in the chamber was adjusted to 70 lux. The mice were all placed in the same environment as that of the chamber 30 minutes prior to the test. Each mouse was placed  
15 individually in the middle of the open field and locomotion was recorded for 60 minutes. The horizontal locomotor activity was judged according to the distance the animal moved. The inner 30 percentage of the open field was defined as the center of the chamber.

20

As a result, the locomotor activities shown by Tg-  
βCTF99/B6 at 7-11 months were similar to those of wild type controls. The locomotor activity displayed by Tg-  
βCTF99/B6 at 14 month was slightly elevated, compared to  
25 that of the wild type controls, though it was not

significant (Fig. 8A). The approaches to the center of the open field (a sign of anxiety) were also similar to those of control mice.

5      <10-2> Rota-rod test

Rota-rod test was performed to evaluate motor coordination and motor learning. Rota-rod consists of a rotating cylinder (4.5 cm in diameter) with a speed controller attached. Mice were placed on the top of the 10 cylinder where they have access to tight grip. Rota-rod was spun at the speed of 5-20 rpm, and the speed was gradually increased. Cut-off time was set as 3 minutes and intertrial interval was 60 minutes. Hang-on time on rod was measured.

15

As a result, motor coordination of the transgenic mice of the present invention at 5.5 months was similar to that of wild type controls. However, motor coordination of the transgenic mice of the present invention at 11 20 months was reduced, compared to that of the wild type controls, though the difference was not significant (Fig. 8B).

<10-3> Morris water maze test

25      Morris water maze test is a hippocampus-dependent

analysis method that depends largely on the capability of an animal to learn and remember the relation between stimulus at a distance and hidden platform for escape (Morris *et al.*, 1982, *Nature*, 297, 701). That is, in this 5 test, forced swimming or the latency to find a hidden platform by taking advantage of spatial indices memorized during placing on the platform was observed. Based on this observation, cognitive function of a mouse was investigated and quantified by comparison of the distance 10 and the time that the mouse swam. In order to investigate memory retention of a mouse, the locations of the entrance to the pool and the hidden platform were changed often, while spatial indices were still located same. Particularly, water maze consisted of a 90 cm-diameter 15 cylinder pool filled with 22°C opaque milky water. A 10 cm-diameter hidden platform was placed in a quadrant 1.5 cm below the surface of the opaque water. The pool was placed in a room with abundant environmental and artificial cues including a window, a chair and posters. 20 In the course of daily testing, mice were admitted successively into each of the quadrants and allowed to swim for 90 seconds maximum. On locating the platform, the animals were permitted to remain on it for 30 seconds before the session was terminated. The latency to find 25 the platform for each of two trials and the average of the

two trials were recorded for each mouse.

As a result, wild type controls at 7, 11 and 14 months could recognize the indices of the hidden platform,  
5 and this achievement improved trial after trial. On the other hand, Tg- $\beta$ CTF99/B6 mice at 7, 11 and 14 months showed longer latency to find the hidden platform, compared to the wild type controls, indicating the cognitive deficits, even though the difference was not very  
10 significant (Fig. 9A-B). In the meantime, swimming speeds of Tg- $\beta$ CTF99/B6 mice at 7, 11 and 14 months were similar to those of age-matched controls (Fig. 9C). Those results indicate that Tg- $\beta$ CTF99/B6 mice show elevated cognitive deficits, compared to the wild type controls.  
15

<10-4> Passive avoidance test

Mice prefer darkness to lightness. When mice are allowed to choose one of the two chambers, one is lighted and the other is dark chamber, they have no hesitation to  
20 go for the dark chamber. Mice are once placed in a lighted chamber and then allowed to move to a dark chamber but a strong electric shock is given then (that is, a training). After the training, when mice are forced to select a chamber to enter, most of wild type mice try to  
25 stay in a lighted chamber without the electric shock,

eventhough unwillingly. Passive avoidance test is designed based on the above idea, and so the test is to investigate learning and memory retention through spatial information such as a lighted and a dark chamber, and an 5 electric shock.

Particularly, the test apparatus of the invention consisted of a brightly lit and a dark compartment (15 x 15 x 15 cm each), each equipped with a shock-grid floor, and a door between the two chambers. During the first day 10 of testing, each mouse was placed in the lighted chamber and left to habituate to the apparatus for 5 minutes, while allowing it to explore the light and dark rooms. On the second day, the mice were placed in the lighted chamber. After 30 seconds, the middle door was opened and 15 the latency for the mouse to enter the dark chamber was measured. When the mouse entered the dark room, the door was closed and two successive electric foot-shocks (100 V, 0.3 mA, 2 seconds) were delivered through the grid-floor. After training, mice were individually replaced in the 20 lighted chamber and the latency to enter the dark chamber was measured.

As a result, pre-shock latency to enter the dark chamber of wild type control mice at 7 - 14 months was 25 similar to that of Tg- $\beta$ CTF99/B6 mice of the present

invention. However, the post-shock latency to enter the dark chamber of Tg- $\beta$ CTF99/B6 mice of the present invention was much shorter than that of wild type control mice (Fig. 9D). The results indicate that the transgenic 5 mice of the present invention show cognitive deficits.

<10-5> Elevated plus maze test

Increased anxiety is a problematic symptom of human AD patients (Folstein and Bylsma, 1999, Alzheimer Disease 10 (Eds by Terry et al.,) 2nd. Lippincott Williams & Wilkins, Philadelphia). So, the present inventors needed to investigate the possibility of increased anxiety by the introduction of APP mutant gene in Tg-APP/B6 transgenic mice. Elevated plus maze apparatus consisted of four arms 15 (30 x 7 cm) made of black Plexiglas, which were placed at right angles to each other and elevated 50 cm above the floor. Two of the arms had 20 cm high walls (enclosed arms), while other two had no walls (open arms). The illumination at the center was adjusted to 40 lux. For 20 the test, the mouse was initially placed at the center of the platform and left to explore the arms for 5 minutes. The number of entries in the open and in the enclosed arms and the time spent in each arm was recorded. Entry into each arm was scored as an event if the animal placed all 25 four paws into the corresponding arm.

As a result, the number of entries into open and enclosed arms for Tg- $\beta$ CTF99/B6 at 7 months was similar to that of age-matched controls. However, the number of 5 entries and the time spent in the open arm for the Tg- $\beta$ CTF99/B6 transgenic mice at 13 months was less than that of age-matched controls. The results indicate that Tg- $\beta$ CTF99/B6 mice of the present invention show increased anxiety (Fig. 10).

10

#### **[Industrial Applicability]**

As explained hereinbefore, unlike the wild type control mice, the transgenic mice of the present invention showed notable cognitive deficits, meaning the impaired 15 memory retention, in Morris water maze test. In addition, in elevated plus maze test, the transgenic mice of the present invention showed increased anxiety. Those results confirmed that the transgenic mice of the present invention showed clinical symptoms of AD better than any 20 other conventional AD animal models. The transgenic mice of the present invention showed age-dependent neuronal loss, which is superior to any known conventional AD animal models. Therefore, the transgenic mice of the present invention are expected to serve as a useful AD

model for the study of AD-related pathogenesis including the study of cognitive deficits.

Those skilled in the art will appreciate that the 5 conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such 10 equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

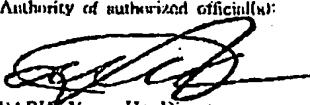
BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT  
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURES

## INTERNATIONAL FORM

**RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT**

Issued pursuant to Rule 7.1

TO : HAN, Pyung Lim  
 Institute of Neuroscience, Ewha Womans University Medical School,  
 #70, Jongno 6-ga, Jongno-gu, Seoul 110 783,  
 Republic of Korea

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR:  -Tg-CFF90/B6 (mouse embryo)	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY:  KCTC 10609BP
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I above was accompanied by: <input checked="" type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depository Authority accepts the microorganism identified under I above, which was received by it on <u>March 10 2004</u> .	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The microorganism identified under I above was received by this International Depository Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____.	
<b>V. INTERNATIONAL DEPOSITORY AUTHORITY</b>	
Name: Korean Collection for Type Cultures	Signature(s) of person(s) having the power to represent the International Depository Authority or authorized official(s):   PARK, Yong-Ha Director Date: March 31 2004
Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-033, Republic of Korea	

**【CLAIMS】****【Claim 1】**

A vector, for transformation of animals to induce Alzheimer's disease pathology, that contains a gene coding 5 for a protein represented by SEQ. ID. No 10 containing C-terminal fragment (CTF) of mutant human amyloid beta precursor protein (APP) in which 698<sup>th</sup> amino valine (V) of AP751 is replaced with phenylalanine (F).

**10 【Claim 2】**

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 1, wherein the vector additionally includes a promoter and polyadenylation region.

15

**【Claim 3】**

The vector design for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 2, wherein the promoter is human PDGF- $\beta$  promoter.

20

**【Claim 4】**

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 2, wherein the polyadenylation region is SV40 pA.

25

**【Claim 5】**

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 2, wherein the vector additionally includes Kozac sequence 5 between a promoter and a gene coding C-terminal fragment of the mutant human amyloid beta precursor protein.

**【Claim 6】**

The vector for transformation of animals to induce 10 Alzheimer's disease pathology as set forth in claim 2, wherein the vector additionally includes nucleotide sequence coding signal peptide in front of a gene coding C-terminal fragment of mutant human amyloid beta precursor protein.

15

**【Claim 7】**

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 6, wherein the nucleotide sequence is represented by SEQ. ID. 20 No 25.

**【Claim 8】**

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 2, 25 wherein the vector is designed to include human PDGF- $\beta$

promoter gene, mutant gene coding an amino acid sequence represented by SEQ. ID. No 3 and SV40 pA in that order, and represented by the cleavage map PDGF- $\beta$ CTF99(V717F)-pA.

5       【Claim 9】

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in any of claim 2 ~ claim 7, wherein the vector additionally includes intron between a promoter gene and a mutant gene coding a 10 mutant protein.

【Claim 10】

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 9, 15 wherein the intron is intron B that is derived from human beta-globin gene.

【Claim 11】

The vector for transformation of animals to induce 20 Alzheimer's disease pathology as set forth in claim 9, wherein the vector is designed to include human PDGF- $\beta$  promoter gene, intron B gene of human beta-globin, mutant gene coding an amino acid sequence represented by SEQ. ID. No 3 and SV40 pA in that order, and represented by the 25 cleavage map PDGF-intron- $\beta$ CTF99(V717F)-pA.

**【Claim 12】**

A transgenic mouse with induced Alzheimer's disease pathology generated by introducing the vector for  
5 transformation of animals of claim 1.

**【Claim 13】**

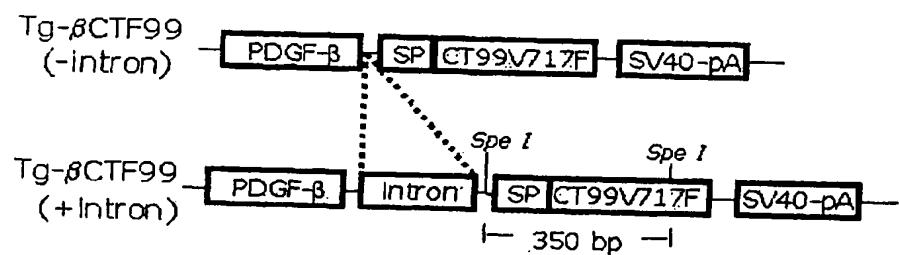
The transgenic mouse with induced Alzheimer's disease pathology as set forth in claim 12, wherein the  
10 mouse is Tg- $\beta$ CTF/B6 showed clinical symptoms of AD such as motor coordination deficit, impaired memory retention, cognitive deficits and increased anxiety (Accession No: KCTC 10609BP).

1/10

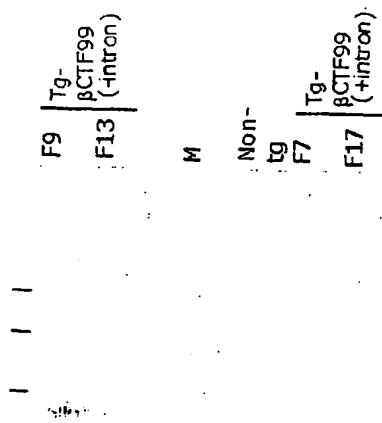
## FIGURES

FIG. 1

A



B

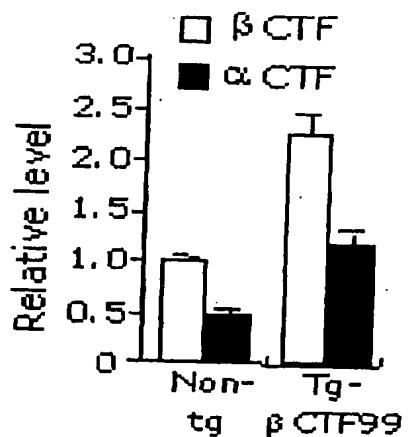
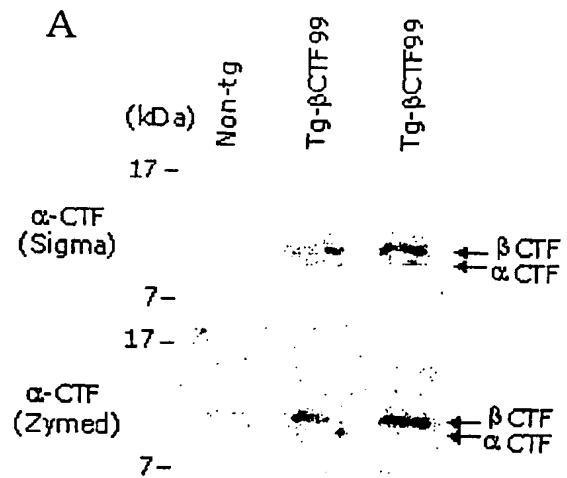


C



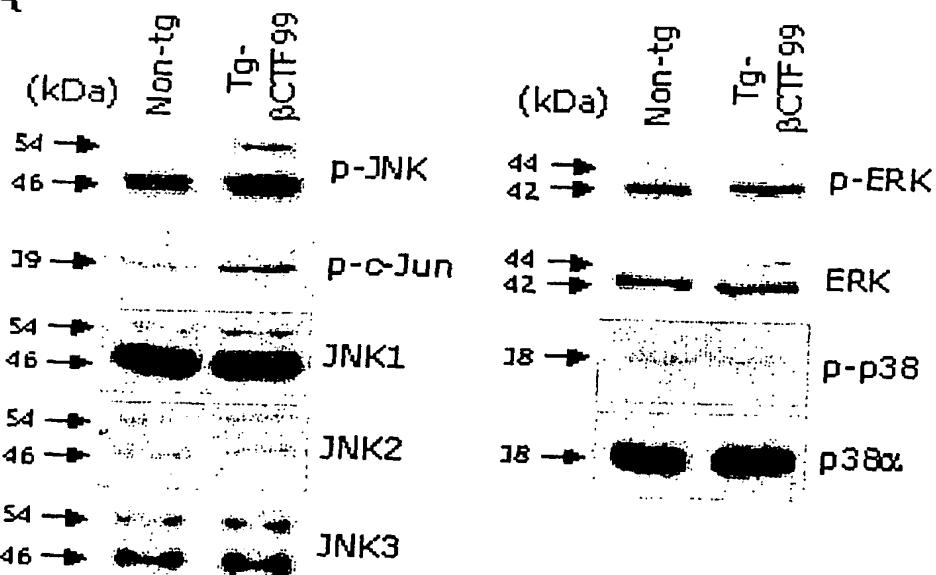
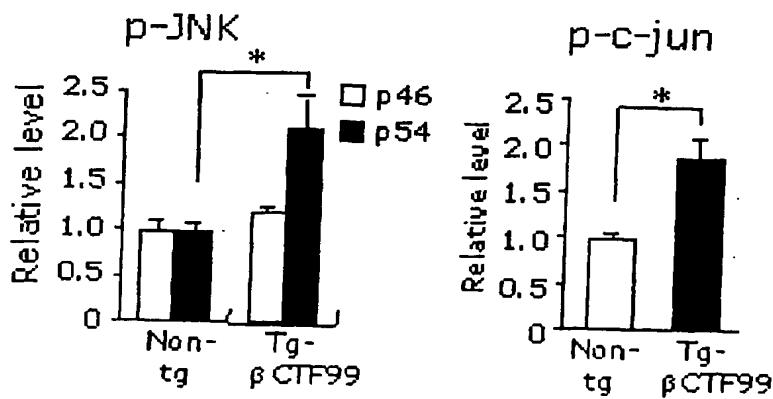
2/10

FIG. 2

**A****B****C**

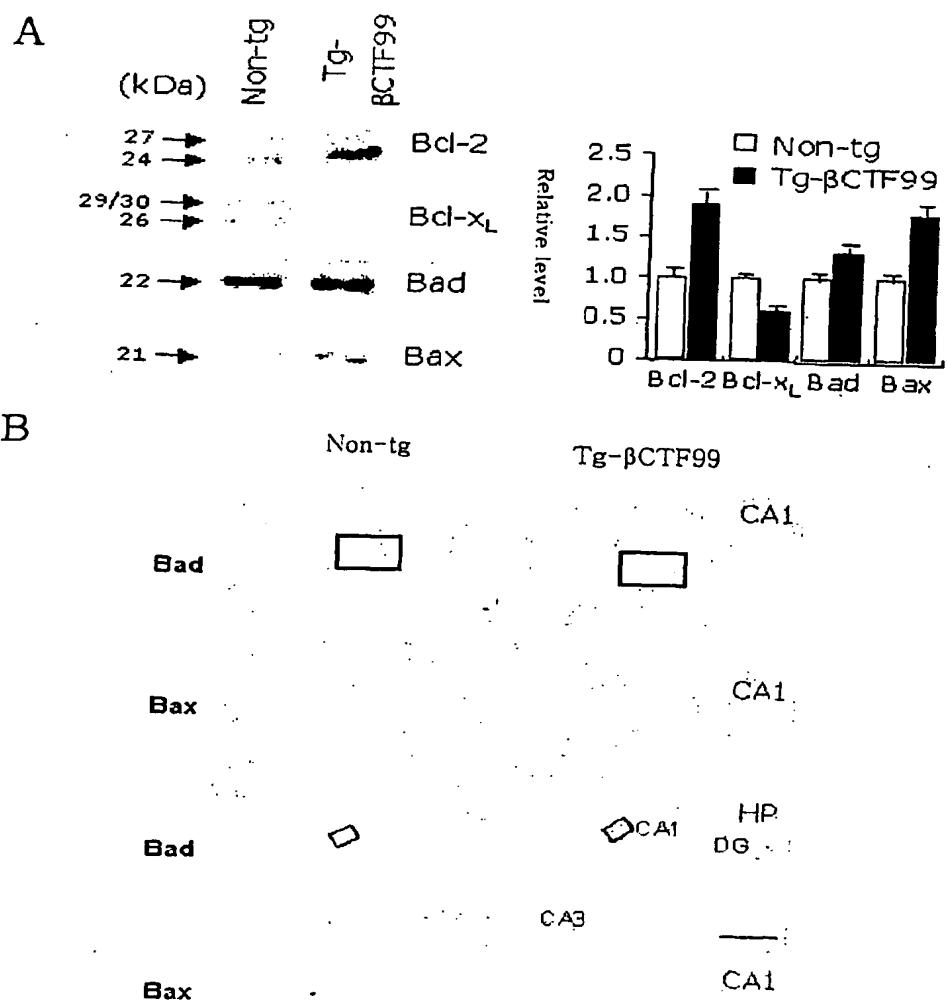
3/10

FIG. 3

**A****B**

4/10

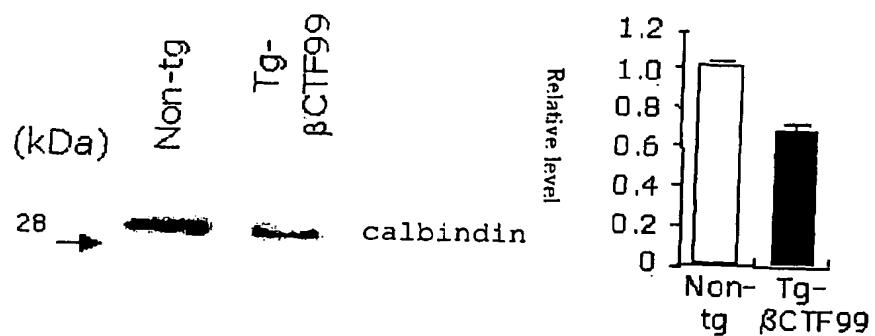
FIG. 4



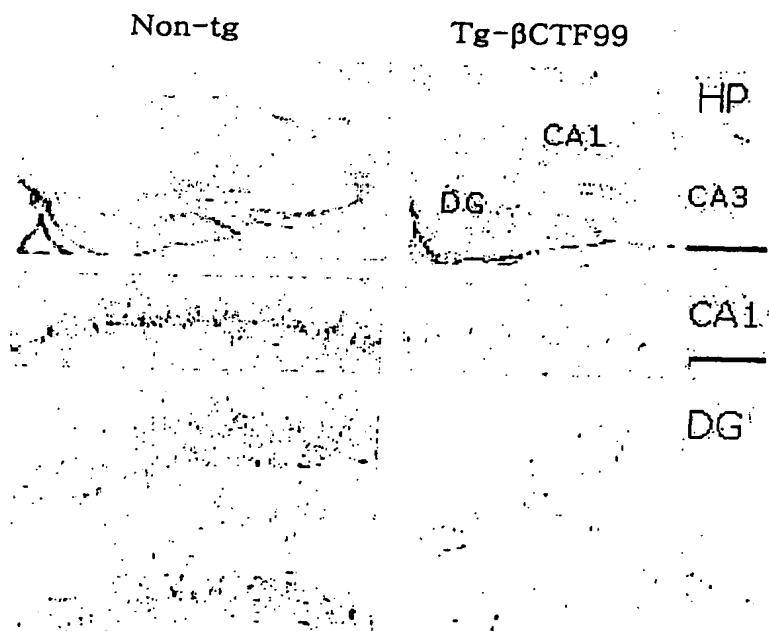
5/10

FIG. 5

A

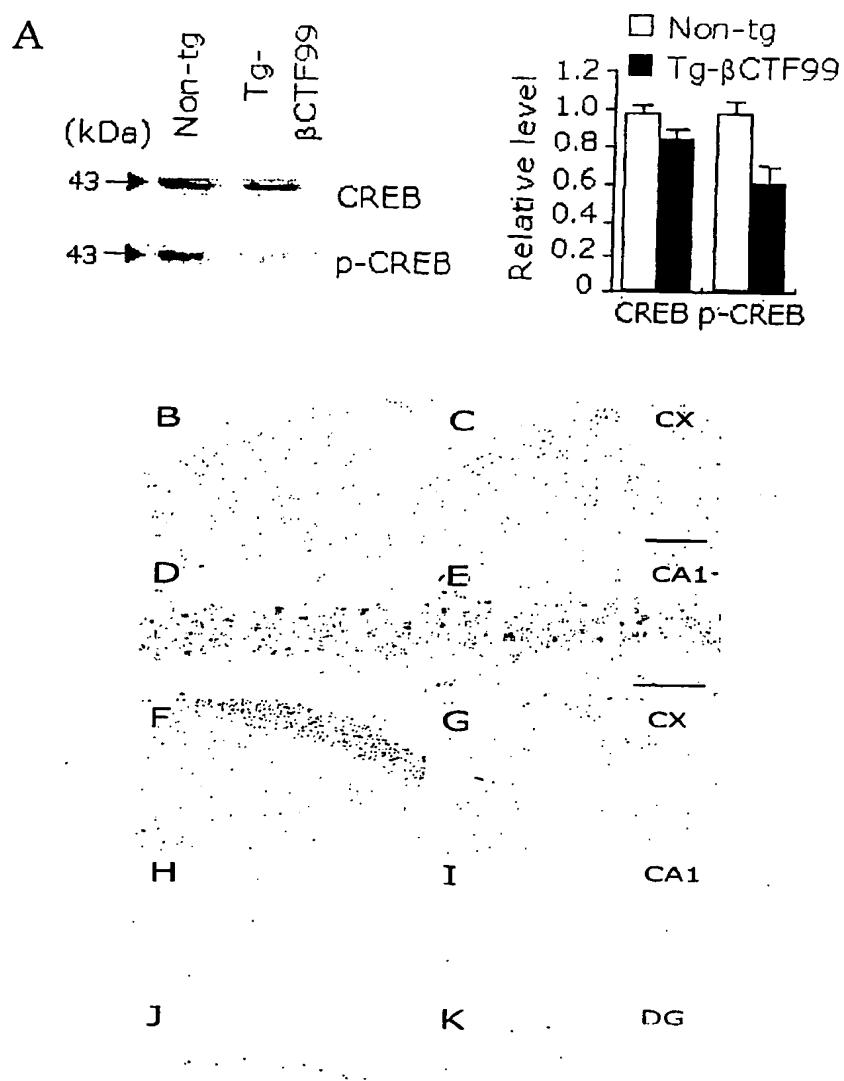


B



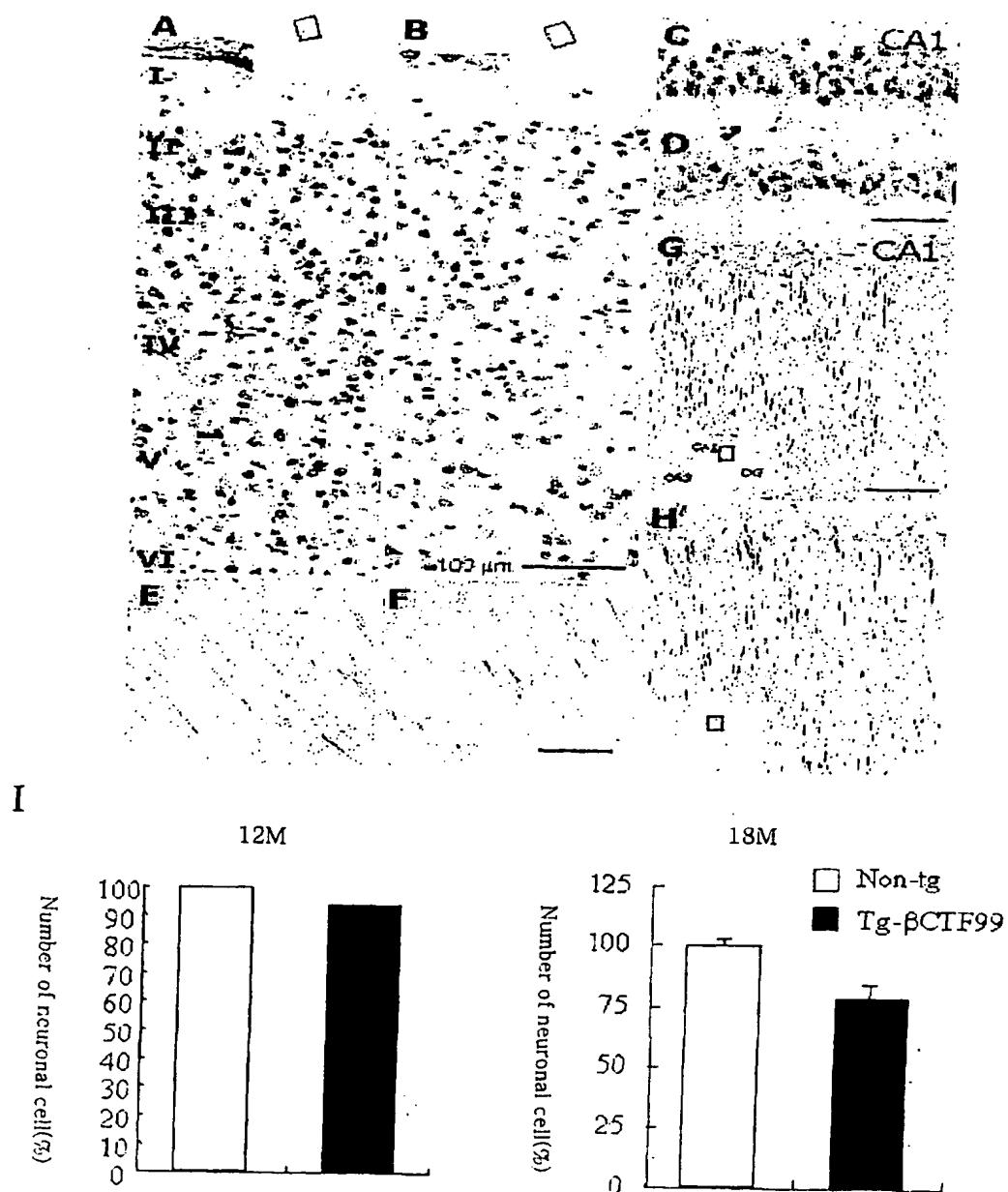
6/10

FIG. 6



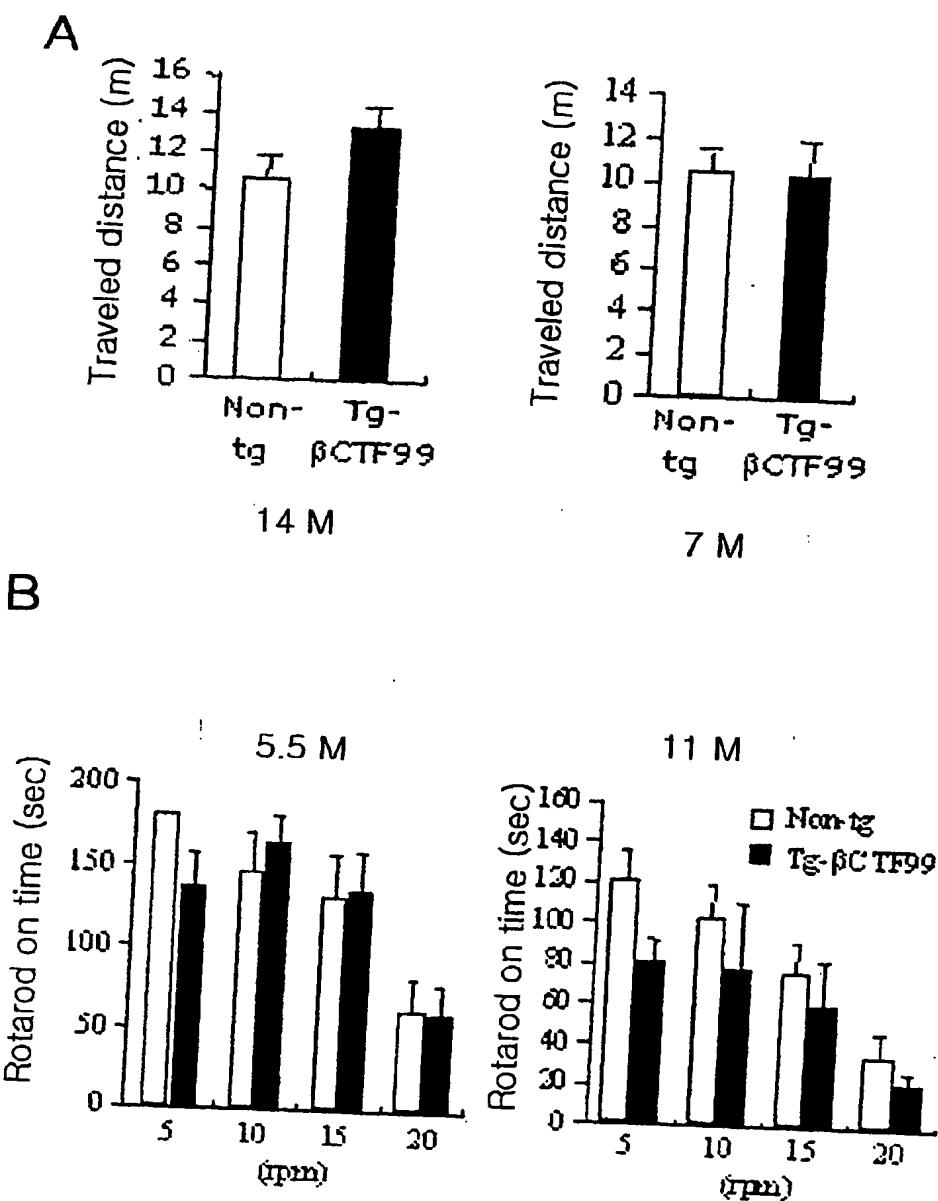
7/10

FIG. 7



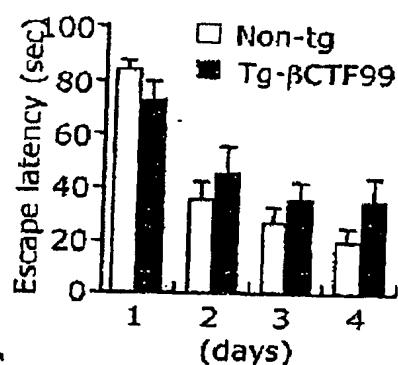
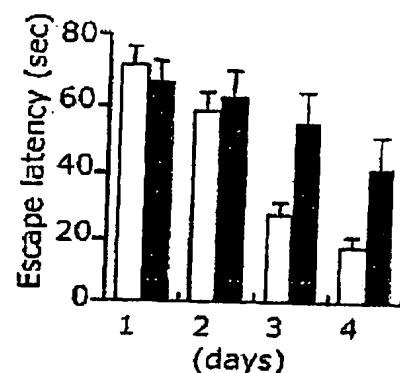
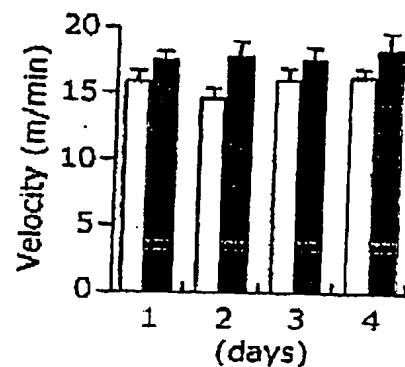
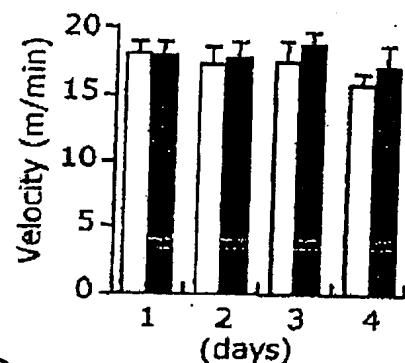
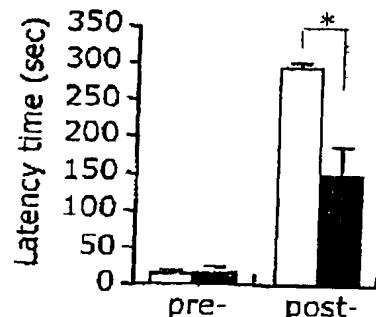
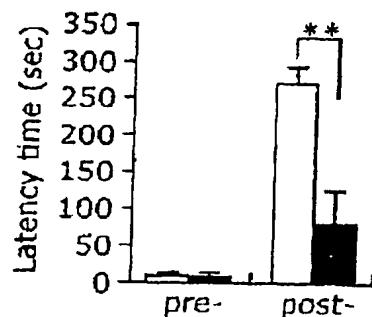
8/10

FIG. 8



9/10

FIG. 9

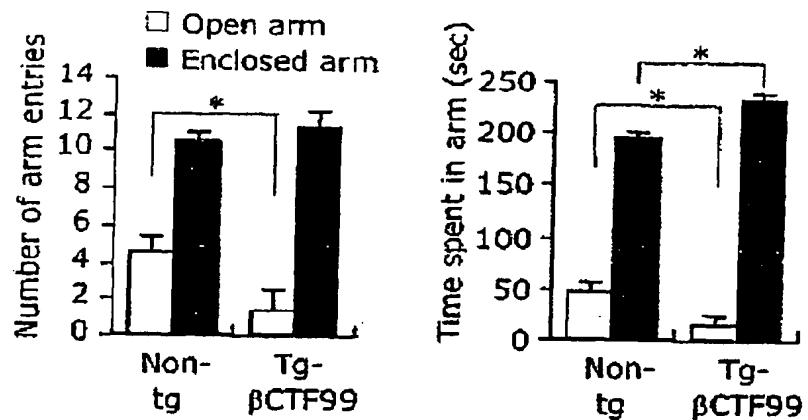
**A****B****C****D**

7M

14M

10/10

FIG. 10



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/KR2005/000969

## A. CLASSIFICATION OF SUBJECT MATTER

**IPC7 C12N 15/63**

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols):

IPC7 C12N 15/63, A01K 67/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
Korean patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
eKIPASS, Delphion, NCBI PubMed, GenBank database

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	R. GERLAJ, ET. AL. "Behavioral impairment of APP(V717F) mice in fear conditioning: is it only cognition?" In: Behav. Brain Res., (November 2002) Vol.136(2):503-509, see the whole document.	1-13
Y	E. M. ROCKENSTEIN, ET. AL. "Levels and Alternative Splicing of Amyloid $\beta$ Protein Precursor (APP) Transcripts in Brains of APP Transgenic Mice and Humans with Alzheimer's Disease." In: J. Biol. Chem., (24 November 1995) Vol.270(47):28257-28267, see the abstract, figure 1, 2, and Materials and Methods.	1-13
Y	B. P. F. RUTTEN, ET. AL. "No alterations of hippocampal neuronal number and synaptic bouton number in a transgenic mouse model expressing the $\beta$ -cleaved C-terminal APP fragment." In: Neurobiol. Dis., (March 2003) Vol.12(2):110-120, see the abstract and Materials and Methods.	1-13
Y	T. KAWARABAYASHI, ET. AL. "Accumulation of $\beta$ -Amyloid Fibrils in Pancreas of Transgenic Mice." In: Neurobiol. Aging, (March-April 1996) Vol.17(2):215-222, see the abstract, figure 1, and Methods.	1-13
A	US 6717031 B2 (KATE DORA GAMES; DALE BERNARD SCHENK; LISA CLAIRE MCCONLOGUE; PETER ANDREW SEUBERT; RUSSELL E. RYDEL) 6 April 2004 (2004-04-06), see the whole document.	1-13

Further documents are listed in the continuation of Box C.

See patent family annex.

- \* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "V" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search  27 JUNE 2005 (27.06.2005)	Date of mailing of the international search report  <b>27 JUNE 2005 (27.06.2005)</b>
Name and mailing address of the ISA/KR   Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea	Authorized officer  AHN, Kyu Jeong
Faxsimile No. 82-42-472-7110	Telephone No. 82-42-181-5026

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/KR2005/C00969

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

## a. type of material

- a sequence listing  
 table(s) related to the sequence listing

## b. format of material

- on paper  
 in electronic form

## c. time of filing/furnishing

- contained in the international application as filed  
 filed together with the international application in electronic form  
 furnished subsequently to this Authority for the purposes of search

2.  In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/KR2005/000969

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S. SINHA AND I. LIEBERBURG. "Cellular mechanisms of $\beta$ -amyloid production and secretion." In: PNAS, (28 September 1999) Vol.96(20):11049-11053, see the whole document.	1-13

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.  
PCT/KR2005/000969

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US06717031	06.04.2004	US20020104104A1 US2004226054A1	01.08.2002 11.11.2004

## PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING  
OF A CHANGE(PCT Rule 92bis.1 and  
Administrative Instructions, Section 422)

Date of mailing (day/month/year)  
02 March 2006 (02.03.2006)

From the INTERNATIONAL BUREAU

To:

LEE, Sang-Yong  
4F., Byukcheon Bldg.,  
1579-5, Seocho-dong,  
Seocho-gu,  
Seoul 137-876  
Republic of Korea

Applicant's or agent's file reference  
5FPO-03-18 PCT 06-006

## IMPORTANT NOTIFICATION

International application No.  
PCT/KR2005/000969

International filing date (day/month/year)  
01 April 2005 (01.04.2005)

## 1. The following indications appeared on record concerning:

the applicant  the inventor  the agent  the common representative

## Name and Address

LEE, Won-Hee  
8th Fl. Sung-ji Heights II 642-16  
Yoksam-dong Kangnam-ku  
Seoul 135-080  
Republic of Korea

## State of Nationality

## State of Residence

## Telephone No.

82-2-3453-0507

## Facsimile No.

82-2-3453-8155

## Teleprinter No.

## 2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

the person  the name  the address  the nationality  the residence

## Name and Address

LEE, Sang-Yong  
4F., Byukcheon Bldg.,  
1579-5, Seocho-dong,  
Seocho-gu,  
Seoul 137-876  
Republic of Korea

## State of Nationality

## State of Residence

## Telephone No.

82-2-588-5757

## Facsimile No.

82-2-588-5790

## Teleprinter No.

## 3. Further observations, if necessary:

The appointment of the agent of record has been revoked. A new agent has been appointed,  
as indicated in Box 2.

## 4. A copy of this notification has been sent to:

the receiving Office  
 the International Searching Authority  
 the International Preliminary Examining Authority

the designated Offices concerned  
 the elected Offices concerned  
 other: LEE, Won-Hee

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Authorized officer

Mehdi FILALI

Facsimile No. +41 22 338 7090

Telephone No. (41-22) 338 8784

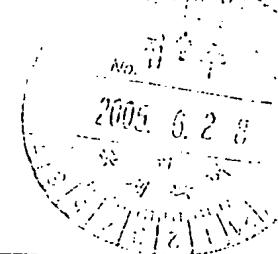
## PATENT COOPERATION TREATY

PCT/KR2005/000969

**PCT**

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)



Applicant's or agent's file reference 5FPO-03-18	<b>FOR FURTHER ACTION</b>	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. <b>PCT/KR2005/000969</b>	International filing date (day/month/year) <b>01 APRIL 2005 (01.04.2005)</b>	(Earliest) Priority Date (day/month/year) <b>01 APRIL 2004 (01.04.2004)</b>
Applicant <b>EWHA UNIVERSITY-INDUSTRY COLLABORATION FOUNDATION et al</b>		

This International search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

## 1. Basis of the report

- a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

The international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b.  With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.

2.  Certain claims were found unsearchable (See Box No. II)

3.  Unity of invention is lacking (See Box No. III)

## 4. With regard to the title,

the text is approved as submitted by the applicant.

the text has been established by this Authority to read as follows:

## 5. With regard to the abstract,

the text is approved as submitted by the applicant.

the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

## 6. With regard to the drawings,

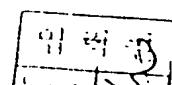
- a. the figure of the drawings to be published with the abstract is Figure No. 1

as suggested by the applicant.

because the applicant failed to suggest a figure

because this figure better characterizes the invention

- b.  none of the figure is to be published with the abstract



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2005/000969

## Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

## a. type of material

- a sequence listing  
 table(s) related to the sequence listing

## b. format of material

- on paper  
 in electronic form

## c. time of filing/furnishing

- contained in the international application as filed  
 filed together with the international application in electronic form  
 furnished subsequently to this Authority for the purposes of search

2.  In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

## 3. Additional comments:

## INTERNATIONAL SEARCH REPORT

international application No.

PCT/KR2005/000969

## A. CLASSIFICATION OF SUBJECT MATTER

**IPC7 C12N 15/63**

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12N 15/63, A01K 67/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
Korean patents and applications for inventions since 1975Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
eKIPASS, Delphion, NCBI PubMed, GenBank database

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	R. GERLAI, ET. AL. "'Behavioral impairment of APP(V717F) mice in fear conditioning: is it only cognition?." In: Behav. Brain Res., (November 2002) Vol.136(2):503-509, see the whole document.	1-13
Y	E. M. ROCKENSTEIN, ET. AL. "Levels and Alternative Splicing of Amyloid $\beta$ Protein Precursor (APP) Transcripts in Brains of APP Transgenic Mice and Humans with Alzheimer's Disease." In: J. Biol. Chem., (24 November 1995) Vol.270(47):28257-28267, see the abstract, figure1, 2, and Materials and Methods.	1-13
Y	B. P. F. RUTTEN, ET. AL. "No alterations of hippocampal neuronal number and synaptic bouton number in a transgenic mouse model expressing the $\beta$ -cleaved C-terminal APP fragment." In: Neurobiol. Dis., (March 2003) Vol.12(2):110-120, see the abstract and Materials and Methods.	1-13
Y	T. KAWARABAYASHI, ET. AL. "Accumulation of $\beta$ -Amyloid Fibrils in Pancreas of Transgenic Mice." In: Neurobiol. Aging, (March-April 1996) Vol.17(2):215-222, see the abstract, figure1, and Methods.	1-13
A	US 6717031 B2 (KATE DORA GAMES; DALE BERNARD SCHENK; LISA CLAIRE MCCONLOGUE; PETER ANDREW SEUBERT; RUSSELL E. RYDEL) 6 April 2004 (2004-04-06), see the whole document.	1-13

 Further documents are listed in the continuation of Box C. See patent family annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 JUNE 2005 (27.06.2005)

Date of mailing of the international search report

27 JUNE 2005 (27.06.2005)

Name and mailing address of the ISA/ER

Korean Intellectual Property Office  
920 Dunsan-dong, Seo-gu, Daejeon 302-701,  
Republic of Korea

Authorized officer

AHN, Kyu Jeong

Facsimile No. 82-42 472 7140

Telephone No. 82-42-481-5026

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2005/000969

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S. SINHA AND I. LIEBERBURG, "Cellular mechanisms of $\beta$ -amyloid production and secretion." In: PNAS, (28 September 1999) Vol.96(20):11049-11053, see the whole document.	1-13

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

PCT/KR2005/000969

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US06717031	06.04.2004	US20020104104A1 US2004226054A1	01.08.2002 11.11.2004

## PATENT COOPERATION TREATY

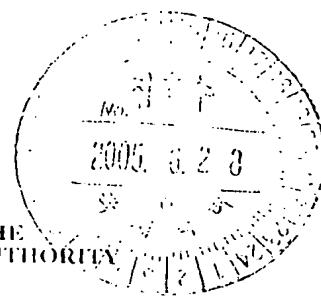
From the  
INTERNATIONAL SEARCHING AUTHORITY

To: LEE, Won-Hee  8th Fl, Sung-ji Heights Ho-42-16 Yoksam-dong Gangnam-ku Seoul 135-080 Republic of Korea
---

PCT

WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)



Applicant's or agent's file reference 5FPO-03-18		Date of mailing (day/month/year) 27 JUNE 2005 (27.06.2005)
International application No. <b>PCT/KR2005/000969</b>	International filing date (day/month/year) <b>01 APRIL 2005 (01.04.2005)</b>	Priority date(day/month/year) <b>01 APRIL 2004 (01.04.2004)</b>
International Patent Classification (IPC) or both national classification and IPC <b>IPC7 C12N 15/63</b>		
Applicant <b>EWHA UNIVERSITY-INDUSTRY COLLABORATION FOUNDATION et al</b>		

## 1. This opinion contains indications relating to the following items:

- Box No. I Basis of the opinion
- Box No. II Priority
- Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- Box No. IV Lack of unity of invention
- Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- Box No. VI Certain documents cited
- Box No. VII Certain defects in the international application
- Box No. VIII Certain observations on the international application

## 2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later. For further options, see Form PCT/ISA/220.

## 3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA, KR  Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea	Date of completion of this opinion 27 JUNE 2005 (27.06.2005)	Authorized officer AHN, Kyu Jeong
Faxsimile No. 82-42-472-7140		Telephone No. 82-42-481-5026

WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/KR2005/000969

Box No. 1 Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.  
 This opinion has been established on the basis of a translation from the original language into the following language , which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).
2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:
  - a. **type of material**  
 a sequence listing  
 table(s) related to the sequence listing
  - b. **format of material**  
 on paper  
 in electronic form
  - c. **time of filing/furnishing**  
 contained in the international application as filed.  
 filed together with the international application in electronic form.  
 furnished subsequently to this Authority for the purposes of search.
3.  In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
4. Additional comments:

**WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/KR2005/000969

**Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Claims	1-13	YES
	Claims	None	NO
Inventive step (IS)	Claims	None	YES
	Claims	1-13	NO
Industrial applicability (IA)	Claims	1-13	YES
	Claims	None	NO

**2. Citations and explanations :**

The following documents have been considered for the purpose of this written opinion:

- D1: Behav. Brain Res., Vol.136(2):503-509
- D2: J. Biol. Chem., Vol.270(47):28257-28267
- D3: Neurobiol. Dis., Vol.12(2):110-120
- D4: Neurobiol. Aging, Vol.17(2):215-222

**1. Novelty and Inventive Step**

The present invention relates to a transgenic animal having Alzheimer's disease. Particularly, the subject matter of claims 1 to 11 relates to a vector for inducing Alzheimer's disease in animal model, containing a carboxyl-terminal fragment of human amyloid precursor (hAPP) which contains mutation V717F( $\beta$ CTF99(V717F)). The subject matter of claims 11 to 13 relates to a transgenic mouse having induced Alzheimer's disease pathology generated by microinjection of the said vector into a pronuclei of a fertilized oocyte followed by generating mice.

D1 discloses a transgenic mouse exhibiting learning and memory performance deficits, and altered emotionality, which overexpresses hAPP carrying the mutation V717F.

D2 discloses a transgene comprising a platelet-derived growth factor promoter, APP carrying the mutation V717F (APPInd), intron, SV40 pA region.

D3 discloses transgenic mice expressing human  $\beta$ -CTF with the I45F mutation under the control of the prion protein promoter.

D4 discloses transgenic mice expressing human  $\beta$ -CTF, which are generated using the transgene of signal peptide and C-terminal 99 residues of APP under the control of CMV enhancer/chicken  $\beta$ -actin promoter.

(Continued on Supplemental Box.)

WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

International application No  
PCT/KR2005/000969

**Supplemental Box**

In case the space in any of the preceding boxes is not sufficient.

Continuation of :

Box V. 2

None of the prior art documents D1 to D4 disclose the vector containing only a C-terminal fragment of hAPP carrying V717F mutation ( $\beta$ CTF99V717F), and a transgenic mouse generated by the same.

Thus, the novelty of the subject matter of claims 1 to 13 can be acknowledged [PCT Article 33(2)].

Since there are transgenic mice expressing  $\beta$ -CTF or  $\beta$ -CTF(I45F) to address the potential neurotoxicity of the  $\beta$ -CTF of hAPP (D3, D4), and D1, D2 disclose that V717F mutation of APP alters proteolytic processing of APP and the mouse expressing APP(V717F) exhibits Alzheimer's disease pathology, it appears to be obvious to a person skilled in the art to generate transgenic mice expressing  $\beta$ -CTF carrying V717F mutation from the teachings of D1 to D4.

The claims 1-13 therefore cannot be regarded as meeting the requirement of inventive step [PCT Article 33(3)].

**2. Industrial Applicability**

The subject matter of claims 1-13 is considered to be industrially applicable [PCT Article 33(4)].